EMMELOES DE MARE – BREDEMEIJER

Determinants of Complications in Liver Transplant Patients

The studies described in this thesis were performed at the Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands.

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DETERMINANTS OF COMPLICATIONS IN LIVER TRANSPLANT PATIENTS

Determinanten van complicaties in levertransplantatie patiënten

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Promotor:	Prof.dr. H.J. Metselaar
Overige leden:	Prof.dr. R.W. Hendriks Prof.dr. A. Verbon Prof.dr. R.J.M. ten Berge
Copromotor:	Dr. J. Kwekkeboom

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CHAPTER 1

General introduction and outline of the thesis

INTRODUCTION

1. Liver function and liver disease

The liver is the largest internal organ in the body, weighing about 1.5 kg, and is situated under the rib cage on the right side of the abdomen. It is divided into right and left lobes by the middle hepatic vein (1). In the liver, nutrients that are absorbed in the digestive tract are processed and stored for use in other parts of the body. It is thus an interface between the digestive system and the blood. Most of its blood (about 70%) comes from the intestine via the portal vein; the hepatic artery supplies the remaining percentage (Figure 1). The position of the liver in the circulatory system is optimal for two of its main functions: 1) gathering, processing, and storage of metabolites and 2) neutralization and elimination of toxic substances. Elimination occurs in the bile, an exocrine secretion product of the liver that is important for lipid digestion in the gut. The third important function of the liver is production of carrier proteins, such as albumin; production of factors involved in blood clotting, synthesis of factors important in systemic immunity, and production of hormones (2).

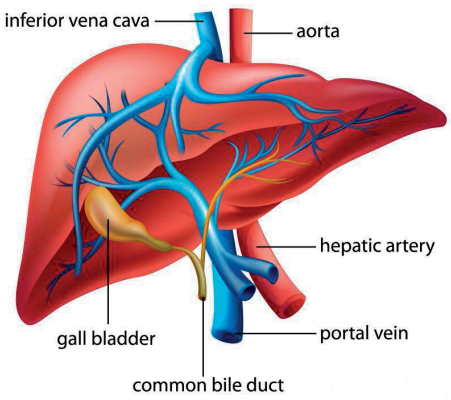

Figure 1. Anatomy of a healthy liver

Since the liver is essential for maintaining body homeostasis, disease of the liver can be life threatening. Over time, damage to the liver due to disease may result in scarring (cirrhosis) which can lead to liver failure, a life-threatening condition. Possible causes of liver failure are: inherited/genetic disorders (such as hemochromatosis or Wilson's disease); immune system abnormalities (such as autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis); infections (such as viral infections with hepatitis A, B, or C); cancer (such as hepatocellular carcinoma or cholangiocarcinoma); chronic

alcohol abuse, or obesity leading to non-alcoholic fatty liver disease; and intoxication, for example with toxic drugs (3).

2. Complications in patients with end-stage liver disease

As described above, liver disease can result in cirrhosis. Cirrhosis is an advanced stage of liver fibrosis that is accompanied by distortion of the hepatic vasculature. It leads to shunting of the portal and arterial blood supply directly into the hepatic outflow (central veins), which compromises exchange of nutrients and oxygen between hepatic sinusoids and the adjacent liver parenchyma (hepatocytes) (4). When cirrhosis progresses and becomes irreversible, it results in a state of end-stage liver disease (ESLD), with liver transplantation (LTx) as the only life-saving treatment option (described in next paragraph). Because of the shortage of donor organs, ESLD patients are waitlisted for liver transplantation, and during their time on the waiting list they may suffer from severe complications of their disease and even die before they can become transplanted. Important complications of cirrhosis are bleeding of esophageal varices, ascites, encephalopathy, renal failure, spontaneous bacterial peritonitis (SBP) and other severe infections (4). Severe bacterial infections (SBIs) represent the second leading cause of death in patients with ESLD waitlisted for LTx (5, 6), with mortality approaching 10% in Europe (7) and 23% in the US (8). Liver dysfunction leads to several abnormalities of the defense mechanisms against pathogens, as both humoral and cell-mediated immunity are suppressed, and bacterial translocation from the intestine increases susceptibility to infection, particularly SBP. Due to infection, a systemic inflammatory response syndrome may occur resulting in sepsis, renal failure, encephalopathy, and death (6).

3. Liver transplantation

Because ESLD may lead to liver failure and life-threatening complications, the diseased liver in patients with ESLD needs to be removed and replaced by a healthy liver coming from another person (most commonly a deceased organ donor). This treatment is called liver transplantation (LTx). Thomas Starzl performed the first successful human LTx in Denver, USA in 1967 (9). Before that, human LTx was an experimental procedure with short patient survival, due to surgical complications and failure to obtain good immediate liver function or the inability to subsequently maintain such function (9). Since the outcome of LTx was not mainly driven by surgical complications or primary non-function anymore, the two most important complications that needed attention became: 1) rejection of the donor liver, also called allograft rejection (“allo-“ means non-self or foreign; “graft” is another word for transplant); and 2) infections early after LTx.

4. Innate immunity and the occurrence of infections in patients before and after liver transplantation

Cirrhotic patients are prone to develop SBIs because of compromised antimicrobial defense caused by several complications of cirrhosis. The liver produces factors important in systemic immunity, which is impaired in patients with cirrhosis, leading to a higher susceptibility to infections. Other reasons for the susceptibility to SBIs are portal hypertension, bacterial translocation from the gut (5, 6, 10, 11), in combination with dysfunction of immune cells (12, 13). Also after LTx, infections are an important complication, being the leading cause of death in the first year after LTx (14, 15). One of the major reasons for this is that patients use immunosuppressive drugs after LTx, which leads to a higher susceptibility to infections. To identify patients before and after LTx who are at high risk for development of infections, insight into the mechanisms of the increased susceptibility for infections is needed. In this paragraph, an introduction is given on the role of the immune system, especially of innate immunity receptors, in the response against infections. In addition, genetic variants in innate immunity receptors will be introduced.

Innate and adaptive immune system

The function of the immune system is to protect the body against invading microorganisms. This system is divided into innate immunity and adaptive immunity. The innate immune system is a relatively non-specific system that in general detects pathogens rapidly (within a few minutes) by recognition of molecular structures, such as certain carbohydrate patterns, that are present on a broad variety of microorganisms, but not on human cells. These structures are called pathogen-associated molecular patterns (PAMPs), and they are detected by pattern recognition receptors (PRRs). One type of PRRs are **signaling** receptors, which are expressed by cells of the innate immune system and, upon ligation to PAMPs, activate them to attack the invading pathogen. The Toll-like receptor (TLR)-family is the best-characterized class of signaling PRRs in mammalian species. Other types of PRRs are: **endocytic** receptors (such as DC-SIGN and mannose receptor), and **secreted** opsonizing receptors (such as Mannose Binding Lectin (MBL) and complement factors). Signaling and secreted PRRs activate the cells of the innate immune system, such as macrophages, dendritic cells (DCs), granulocytes, and Natural Killer (NK) cells, to kill the invading microorganisms or the host cells infected by pathogens (16).

The adaptive immune system is a specific system that responds very specific to antigens and generates long-lasting memory of the specific antigen, thereby providing antigen-specific protection. Though, this response takes longer (3-5 days) than the response of the innate immune system. However, by induction of memory cells, the adaptive immune system can generate more rapid and effective responses to subsequent exposures to the antigen. The adaptive immune system is mediated by T cells and B cells. Adaptive immune responses are initiated and regulated by innate immune cells.

Antigen-presenting cells (APCs), such as DCs and macrophages, activate antigen-specific T-cells; and neutrophils and NK-cells regulate T-cell and B-cell migration. Thus, whereas the innate immune response generates fast but relatively non-specific responses, it activates the adaptive immune system to provide more specific and persistent immune responses (16, 17).

Toll-like receptors and other innate immunity receptors

Humans have 10 TLRs and these are expressed on macrophages, neutrophils, NK cells and DCs and detect multiple PAMPs. An overview of TLRs is provided in Table 1. After detecting PAMPs, most TLRs use myeloid differentiation primary response protein (MyD88) as the signal adapter, while TLR3 uses Toll/interleukin 1 receptor (TIR)-domain-containing adapter-inducing IFN-β (TRIF) (18-20). This initiates a signaling, leading to translocation of the transcription factor NF-κB. In APCs this signaling initiates a maturation program consisting of increased expression of co-stimulatory molecules, and release of pro-inflammatory cytokines and chemokines, which together improve their capacity to activate T cells. Thus, signaling PRRs not only activate innate immune cell defense mechanisms such as phagocytosis or direct cytotoxic killing, but also shape adaptive immune responses. Especially DCs play an important role in bridging the innate and adaptive immune system. DCs take up antigens, become activated and migrate to local lymphoid tissues where they present antigens to T cells and activate them. TLRs expressed by DCs play an important role in recognition of pathogens, and thereby activation of T cells (17, 21). Chemokines secreted by innate immune cells activated via TLRs bind to the luminal surface of the vascular endothelium and trigger immigration of

Table 1. Toll-like receptors (TLRs) and the pathogen-associated molecular patterns (PAMPs) that each receptor recognizes

TLR	PAMP
TLR1	Microbial lipopeptides present in many bacteria, fungi, parasites and viruses
TLR2	Lipoteichoic acid, peptidoglycan, and lipoproteins of Gram-positive bacteria, lipoarabinomannan of Mycobacteria, and zymosan of Candida, among others
TLR3	Double-stranded RNA of viral origin
TLR4	Gram-negative bacterial lipopolysaccharide (LPS), fungal mannans, and certain viral glycoproteins
TLR5	Flagellin of flagellated bacteria
TLR6	Bacterial lipoprotein
TLR7	Single-stranded RNA
TLR8	Single-stranded RNA
TLR9	Bacterial and viral nucleic acids containing CpG motifs
TLR10	Unknown

Based on Sanclemente et al. World Journal of Gastroenterology 2014

other immune cells including B cells and T cells. To provide maximum surveillance for infectious agents, in addition to the inflammation-induced cell recruitment, most tissues of the body are interlaced with resident innate leukocytes such as DCs, macrophages and mast cells. Pathogen recognition through TLRs on these innate leukocytes regulates the recruitment of other leukocytes to the site of infection by activating tissue stromal cells, tissue-resident innate cells and circulating leukocytes. For some TLRs, accessory molecules are required to initiate signal transduction upon ligand binding. For example, TLR4 is activated by the binding of bacterial Lipopolysaccharide (LPS), but it needs three accessory proteins for signaling to be initiated. LPS is bound by the soluble LPS-binding protein (LBP) and transported to a receptor complex on the cellular membrane, consisting of CD14, TLR4 and MD2. Upon association, this complex triggers TLR4 to signal, thereby activating NF-κB translocation to the nucleus, which in turn activates genes involved in defense against infection (Figure 2).

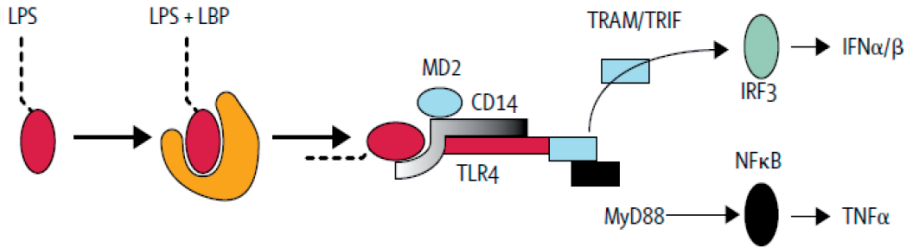

Figure 2. Toll-like receptor 4 (TLR4) pathway. Bacterial lipopolysaccharide (LPS) is bound by LPS binding protein (LBP) and transferred to a receptor complex consisting of CD14, TLR4, and the adapter molecule MD2. The Toll/interleukin 1 receptor (TIR) domain of TLR4 interacts with myeloid differentiation primary response protein (MyD88), initiating a signal transduction cascade leading to the release of pro-inflammatory cytokines—e.g., tumour necrosis factor (TNF)-α via the transcription factor nuclear factor (NF)κB. A second, MyD88-independent, pathway involving TIR domain-containing adapter inducing interferon beta (TRIF) and TRIF-related adaptor molecule (TRAM) leads to the induction of type 1 interferons (IFN) via interferon regulatory factor (IRF) 3. *Adapted from the original figure of Schroder and Schumann, The Lancet infectious diseases, 2005*

Secreted PRRs function as opsonins by binding to microbial cell walls and tagging them for recognition by the complement system and phagocytes. One of the best characterized secreted PRRs is MBL. MBL not only binds to microbial carbohydrates (mannose) to initiate the lectin pathway of complement activation, but also binds phospholipids, nucleic acids and non-glycosylated proteins. These properties may be relevant to the clearance of apoptotic cells and avoidance of autoimmunity (22).

Endocytic PRRs promote the attachment and destruction of microorganisms by phagocytes, without relaying an intracellular signal. These PRRs recognize carbohydrates

and include mannose receptors present on macrophages, glucan receptors present on all phagocytes and scavenger receptors that recognize charged ligands. They are found on all phagocytes and mediate removal of apoptotic cells (16).

Genetic polymorphisms in innate immunity receptors

Genetic polymorphisms - for the most part, single nucleotide polymorphisms (SNPs) - are common variants within a population that are found at a frequency of over 1%. SNPs in coding regions may alter the amino acid sequence (non-synonymous) or alter nucleotides without affecting the encoded amino acid (synonymous), or can induce stop codons and thereby result in synthesis of truncated proteins. SNPs in non-coding regions may affect protein expression, e.g. SNPs in promotor regions may affect promoter functions. Mutations do not occur randomly within the genome, but rather depend on the particular genomic region, as well as on selective pressure. Several SNPs have been described within the genes involved in immune recognition, including genes encoding pattern recognition molecules (23). As described above, infections represent an important complication in waitlisted patients and patients after LTx. SNPs in innate immunity receptors have been identified as risk factors for infections in critically ill patients (20, 23-26). In addition, SNPs in the TLR4 pathway have been described to affect levels of pro-inflammatory cytokines such as IL-1 and TNF-alpha, and increase the risk of infections in non-cirrhotic patients (27-30). However, the role of these SNPs in the occurrence of infections in patients before and after liver transplantation has not been established yet. The adaptive immune system is suppressed by immunosuppressive drugs used in patients after LTx. Genetic variants in the innate immune system may therefore lead to a higher risk of infections in these patients. **Aim 1** of this thesis is therefore to assess whether genetic polymorphisms in innate immunity receptor pathways are associated with infections in patients before and after LTx.

5. Liver allograft rejection and T-cell alloresponses

Besides the occurrence of infections, acute rejection of the liver allograft is another important complication after LTx. Rejection is caused by an immune response of recipient immune cells, primarily T cells, to alloantigens in the graft. These alloantigens are proteins that vary from individual to individual and are perceived as foreign by the recipient, also called polymorphic proteins (16). Between 0.1% and 10% of an individual's T-cell repertoire react with alloantigens (31-35) while only <1/100 000 T cells react to nominal peptide antigens (36). The enormous strength of alloreactive T-cell responses has led to many theories regarding how and why T cells respond to foreign tissues (37, 38), but it was the discovery of the T cell receptor (TCR) (39) and the description of the crystal structure of major histocompatibility complex (MHC) molecules (40) in the 1980s that provided the important structural clues to deciphering allorecognition on a mo-

lecular level. In contrast to a T-cell response to a pathogen in which pathogen-derived peptides are presented on self-MHC molecules to T cells, an allograft expresses its own set of intact allogeneic MHC molecules, which are recognized by recipient T cells on the surface of donor APCs. This is called the 'direct pathway' of allorecognition, and it is caused by cross-reactivity of TCRs that recognize pathogenic peptides presented by self-MHC molecules to allogeneic MHC molecules (41, 42). The large numbers of circulating T cells that cross-react to alloantigens mediate the strong acute rejection responses to allografts in the absence of adequate immunosuppressive therapy. Recipient T cells can also indirectly recognize peptides derived from polymorphic donor proteins presented on recipient MHC molecules. This is called the 'indirect pathway' of allorecognition. In the indirect pathway, recipient APCs that traffic through the allograft phagocytose allogeneic proteins shed by donor cells (the most important allogeneic peptides are those derived from allogeneic MHC molecules) and present them on recipient MHC molecules to recipient T cells (42). In Figure 3 the direct and indirect pathway of allorecognition are shown schematically.

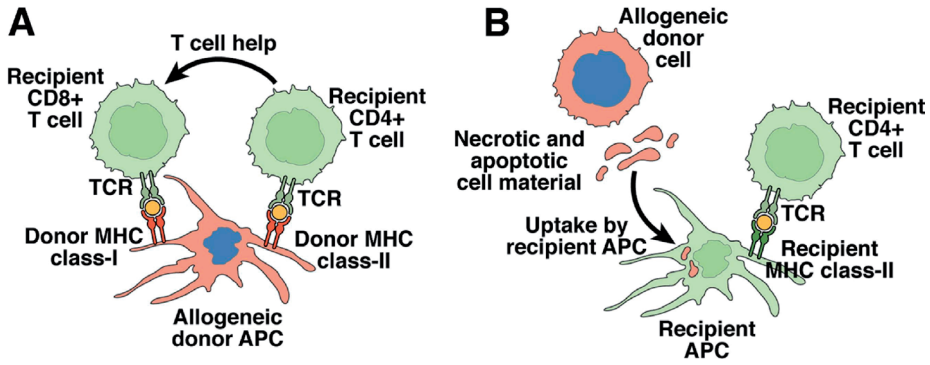

Figure 3. Two non-mutually exclusive pathways of allorecognition: the direct and indirect pathway. (A) In the **direct** pathway, recipient T cells recognize intact allogeneic MHC molecules on the surface of donor antigen presenting cells (APCs). The direct pathway is responsible for the large proportion of T cells that have reactivity against alloantigens due to cross-reactivity of the T-cell receptors (TCR) recognizing pathogenic peptides presented in self MHC to intact foreign MHC molecules. (B) In the **indirect** pathway, recipient APCs trafficking through the allograft phagocytose allogeneic polymorphic proteins shed by donor cells (the most important peptides are those derived from allogeneic MHC molecules) and present them on recipient MHC molecules to recipient T cells. *Adapted from the original figure of Sanchez-Fueyo, Gastroenterology 2011.*

Factors that regulate T-cell alloresponses

T-cells that mediate allograft rejection (through "T-cell alloresponses") require 3 signals to become activated (16). This is shown schematically in Figure 4. The first signal is triggering of the TCR by alloantigen, either directly or indirectly, as described above. The

second signal is the balance between co-stimulatory and co-inhibitory receptors on T cells that regulates T-cell function. The discovery of CD28 as a prototype co-stimulatory receptor provided evidence in the 1970s and '80s for the two-signal model of T-cell activation, according to which both TCR and co-stimulatory signaling are required for full T-cell activation (43-45). Since then, T cell co-signaling receptors have been broadly defined as cell-surface molecules that can either activate (co-stimulatory receptors) or inhibit (co-inhibitory receptors) T-cell responses (46). Other examples of co-stimulatory receptors that can provide signal 2 for T-cell activation are Inducible T-cell co-stimulator (ICOS), which is also a member of the CD28-superfamily, and members of the TNF receptor family (16). The role of co-inhibitory receptors in T-cell responses is described below in more detail. The third signal of T-cell activation is the production of pro-inflammatory cytokines (Interleukin-12 (IL-12), IL-23 and Tumor Necrosis Factor-alpha (TNF-α)) and anti-inflammatory cytokines (IL-10 and Transforming Growth Factor-beta (TGF-β)) by APCs. These cytokines determine differentiation of naïve T cells to pro-inflammatory Th1 or Th17 cells or to anti-inflammatory regulatory T cells, thereby critically contributing to transplant rejection (47). After activation of T cells, antigen-specific T cells proliferate and differentiate into effector T cells that attack the allogeneic tissue. The majority of

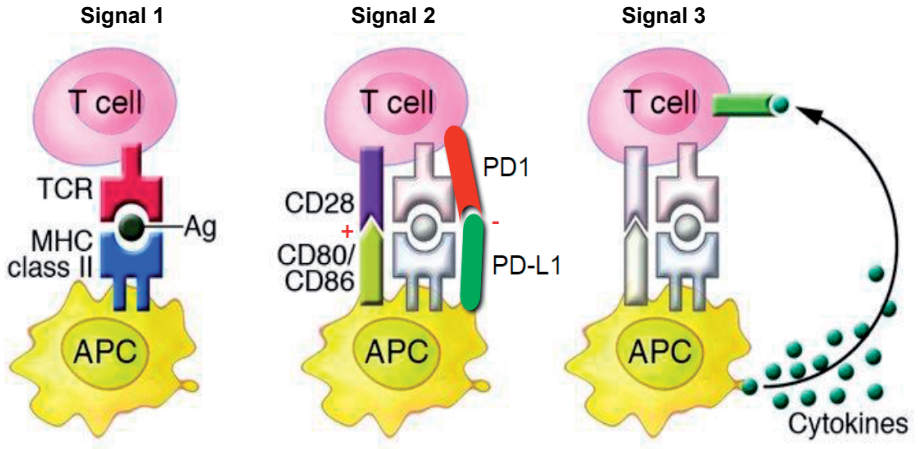

Figure 4. Within the immune synapse formed between antigen presenting cells (APCs) and T cells, three signals are required for antigen-specific T-cell activation. ***Signal 1*** comprises the presentation of antigen (Ag) peptide, in the context of MHC class II molecules, which is recognized by the antigen-specific T-cell receptor (TCR). ***Signal 2*** involves the stabilization of the synapse through adhesion molecules and the generation of signals via costimulatory molecules present on the surface of APCs and T cells. CD80/CD86 on APCs interact with their receptor, CD28, on T cells to generate activating signals, while interaction between Programmed Death 1 (PD1) and its ligand PD-L1 generates inhibitory signals. ***Signal 3*** is produced by the secretion of cytokines by APCs, which signal via cytokine receptors on T cells in order to polarize them towards an effector phenotype. *Adapted from the original figure of Gutcher et al. J Clin Invest. 2007*

effector T cells undergoes apoptosis, but a minority survives and becomes long-lived memory T cells. Memory T cells have been called "a hurdle to immunologic tolerance" in the field of transplantation (48).

Co-inhibitory receptors

As described above, the balance between co-stimulatory and co-inhibitory receptors regulate the outcome of T-cell activation. After solid organ transplantation, blocking of co-stimulatory receptors, for example blocking CD28 with Belatacept, has been shown to inhibit allogeneic T-cell responses and prevent acute and chronic rejection (49-53). Also co-inhibitory receptors expressed on T cells affect T-cell activation and they have been described to inhibit T-cell responses in patients with chronic viral infections (54). Co-inhibitory receptors that have been described to be important in inhibiting T-cell responses are: Programmed Death 1 (PD1), Lymphocyte-Activation Gene 3 (LAG3), T cell immunoglobulin mucin 3 (TIM3), CD160 and CD244 (54, 55). PD1 is a member of the Immunoglobulin superfamily (IgSF) and has two ligands: PD-L1 and PD-L2 (56, 57). PD-L1 is expressed on both hematopoietic cells (T cells, B cells, dendritic cells (DC), macrophages, regulatory T cells (Treg)) and nonhematopoietic cells (parenchymal cells of many organs including liver; and endothelial cells). PD-L2 expression is restricted to DC and macrophages. Binding of PD1 to its ligand(s) negatively regulates T-cell responses (57, 58). LAG3 is a protein closely related to CD4, and mediates negative regulation through interactions with its ligand MHC class II, to which it binds with higher affinity than CD4 (59, 60). TIM3 is expressed on virus-specific T cells of patients with chronic viral infections and inhibits $CD8^+$ T-cell responses in these patients by interaction of TIM3 with its ligand galectin 9 (61, 62). CD160 is a glycosylphosphatidylinositol-anchored receptor that inhibits T-cell responses upon binding with its ligand herpes virus entry mediator (HVEM) that is expressed on both hematopoietic (T cells, B cells, DC, Tregs, monocytes, neutrophils and natural killer (NK) cells) and non-hematopoietic cells (parenchymal cells) (63). CD244, also called 2B4, is a transmembrane receptor of the IgSF primarily expressed by NK cells and antigen-experienced $CD8^+$ T cells that can mediate both activating and inhibitory signals upon binding with its ligand CD48. High levels of CD244 expression are associated with inhibitory receptor function (64-66). Various experimental studies in organ transplanted mice have shown enhanced rejection and/or decreased graft survival after blockade of co-inhibitory receptors. This implies that co-inhibitory receptors are involved in suppressing allograft rejection in mice (67-70). However, the role of co-inhibitory receptor-ligand interactions in human solid organ transplantation has not yet been widely studied (69). **Aim 2** of this thesis is therefore to assess whether co-inhibitory receptors are induced after LTx and whether they inhibit allogeneic T-cell responses in humans. In Table 2 an overview is provided of co-inhibitory receptors and their ligands

that have been described to inhibit T-cell responses during chronic viral infections, and that we studied in this thesis.

Table 2. Co-inhibitory receptors and their ligands

Receptor	Ligand(s)	Remarks
Programmed Death 1 (PD1)	PD-L1 and PD-L2	PD-L1 is expressed on both hematopoietic cells (T cells, B cells, dendritic cells (DCs), macrophages, regulatory T cells (Treg)) and nonhematopoietic cells (parenchymal cells of many organs including liver; and endothelial cells). PD-L2 expression is restricted to DCs and macrophages
Lymphocyte-Activation Gene 3 (LAG3)	MHC class II	LAG3 binds to MHC class II with higher affinity than CD4
T cell immunoglobulin mucin 3 (TIM3)	Galectin 9	
CD160	Herpes virus entry mediator (HVEM)	HVEM is expressed on both hematopoietic (T cells, B cells, DC, Tregs, monocytes, neutrophils and natural killer (NK) cells) and non-hematopoietic cells (parenchymal cells)
CD244, also called 2B4	CD48	CD244 can mediate both activating and inhibitory signals upon binding with its ligand CD48. High levels of CD244 expression are associated with inhibitory receptor function

Based on Wherry EJ. T cell exhaustion. Nat Immunol. 2011

Cytomegalovirus infection (CMV)

Besides expression of co-inhibitory receptors, virus infections after LTx may also affect the outcome of T-cell alloresponses, and thereby influence the risk of rejection after LTx. Cytomegalovirus (CMV) is a prevalent β-herpesvirus that resides in the human body as a latent virus. After LTx and other solid organ transplantations, CMV is the most common viral infection (71), especially in patients who did not experience a CMV infection before LTx and were transplanted with a CMV-seropositive liver transplant donor. This is called "CMV-mismatch" between donor and recipient. T-cell responses to viruses, including CMV, have been proposed as one of the main barriers to achieve transplant tolerance (72), as shown in experimental animal models (73-75). As reviewed by D'Orsogna et al (76), large amounts of virus-specific T cells cross-react against allogeneic HLA, which causes T-cell responses against the donor organ. However, associations between CMV infection and graft rejection in humans vary between different types of organ transplants and show conflicting results (77). Importantly, it is unknown how CMV infection affects T-cell alloresponses after LTx. **Aim 3** of this thesis investigate this possible relation.

6. Immunosuppression after liver transplantation

To prevent allograft rejection, the majority of liver transplant recipients needs to use immunosuppressive drugs life-long. Strikingly however, while almost all patients after other solid organ transplantations need immunosuppressive drugs, in a small percentage of patients after LTx immunosuppressive drugs can be weaned without losing their graft due to rejection. Moreover, HLA –matching between donor and recipient is not necessary and chronic rejection is less frequent after liver transplantation than after other solid organ transplantations, such as kidney and heart transplantation (78). The liver is therefore considered an immune privileged organ (79-81). This so called tolerogenic environment of the liver means that intra-hepatic immune responses are strictly regulated. From a physiological perspective this is beneficial in order to avoid unnecessary inflammation in response to the great amount of bacterial and food antigens derived from the intestines that enter the liver. However, as a consequence, the tolerogenic environment of the liver also negatively impacts immunological responses to hepatotrophic viruses. Likely, the tolerogenic environment of the liver may also dampen the severity of the alloresponse to the liver graft after transplantation.

Several studies in almost 900 patients have shown that approximately 20% of liver transplant recipients can be safely weaned from immunosuppressive drugs and maintain good liver graft function without rejection (82-92). This phenomenon is called 'operational tolerance'. The mechanisms underlying this phenomenon are however not well understood, and it is therefore not possible yet to identify patients in which immunosuppressive drugs can safely be withdrawn (93). Until reliable diagnostic tests are available to predict operational tolerance in liver transplant patients, most patients need to use immunosuppressive drugs life-long.

The currently most used immunosuppressive drug regimen after LTx consists of the Calcineurin Inhibitor (CNI) tacrolimus, combined with prednisolone, and in some centers an induction therapy with IL-2 antagonist basiliximab or dacluzimab. A detailed historic and mechanistic overview of the use of immunosuppressive drugs after LTx is provided in **Chapter 7** of this thesis.

7. Complications of the use of immunosuppressive drugs after liver transplantation

The use of CNIs may have serious side effects, such as infections, nephrotoxicity, diabetes mellitus, hypertension and cancer. Firstly, nephrotoxicity is one of the most serious complications of the use of CNIs (94, 95). Apart from intestinal transplants, liver transplant recipients have the highest five-year incidence of chronic renal failure (CRF) of any non-renal solid organ transplant recipient; additionally, the risk of death is at least

fourfold higher in patients who develop CRF (96). Secondly, CNIs are related with new-onset diabetes mellitus after transplantation, which may lead to severe co-morbidities such as cardiovascular diseases (97, 98). Thirdly, the long-term use of immunosuppressive agents has been associated with an increased risk of developing *de novo* cancer. A recent study in 385 patients transplanted at the Erasmus MC, Rotterdam demonstrated a 2.2-fold higher incidence of *de novo* cancer in LTx patients than in the general population. The cumulative incidences at 1, 5, 10 and 15 years after liver transplantation were 2.9%, 10.5%, 19.4% and 33.6% respectively (99). Finally, infections represent an important complication of the use of immunosuppressive drugs after LTx, which has been discussed in paragraph 4 of this Chapter. Besides recurrence of liver disease, the most common reasons of mortality long after liver transplantation are infection, malignancy, renal failure and cardiovascular events (100).

Despite its potential adverse effects, treatment with tacrolimus still remains the cornerstone of preventing rejection after LTx. The occurrence of these adverse effects has led to a shift in focus from acute cellular rejection and short-term post-transplant survival to long-term management of complications. However, treatment of these complications is often challenging, because in many patients these complications are irreversible and may lead to death (101). It is therefore needed to optimize the use of immunosuppression after LTx. **Aim 4** of this thesis is to describe strategies to optimize currently used immunosuppressive drugs and experimental cell-based immunosuppressive treatment options.

8. Alternative options for immunosuppressive treatment after liver transplantation

Because the currently used immunosuppressive drugs have serious side effects, alternative immunosuppressive treatment strategies are being developed. These include use of intravenous immunoglobulins (102-104) and cell-based immunosuppressive therapies, such as the use of plasmacytoid dendritic cells (105) or Mesenchymal Stem/Stromal Cells (MSCs) (106). The development of these alternative treatment strategies is however still in an experimental phase. **Chapter 8** of this thesis focuses on the immunosuppressive properties of MSCs that are present in liver grafts. MSCs are rare, non-hematopoietic cells that reside in the bone marrow (BM) cavity. They are characterized by their ability to produce colony forming unit-fibroblasts (CFU-F); to support the hematopoietic microenvironment; to promote bone formation and to adhere to plastic *in vitro* (107-109). Besides their presence in the BM, MSC-like cells are present throughout the body at perivascular locations (110, 111) and are critically involved in maintaining tissue homeostasis via anti-apoptotic and tissue-supporting properties (112-114). Importantly, MSCs can suppress T-cell responses (115-117). Bone marrow MSCs (BM-MSCs) can suppress recipient alloreactive T-cell responses and thereby prevent graft rejection (106). Hence, administration

of MSCs seems to be suitable for clinical application in human organ transplantation due to the anti-inflammatory nature of MSCs. Interestingly, human liver grafts also contain MSCs (L-MSCs) (118, 119) and these cells become mobilized from the liver graft during the transplantation procedure. In **Chapter 8** we compare the immunosuppressive properties of BM-MSCs and L-MSCs to assess whether L-MSC in the future may be clinically applicable as an alternative immunosuppressive therapy after LTx.

AIM AND OUTLINE OF THE THESIS

Most liver transplant patients develop one or more complications such as infections, *de novo* malignancies or renal failure related to the immunosuppressive therapy. These complications strongly affect survival and quality of life in these patients. It is therefore urgently needed to develop strategies to predict the risk of these complications and optimize immunosuppression after LTx in order to prevent complications or to treat them in an early stage. Moreover, patients with end-stage liver disease awaiting a liver transplant frequently develop severe infections, sometimes causing death before a donor liver is offered.

The **aim of this thesis** is to identify risk factors for the occurrence of complications in liver transplant patients, both before and after LTx, and develop strategies to optimize immunosuppression after LTx. The thesis is divided into four parts (I-IV), from which the first two parts focus on genetic and viral risk factors for complications before and after LTx; the third part describes different strategies to optimize immunosuppression after LTx, and in the fourth part the findings of this thesis will be summarized and discussed.

Part I (**Chapters 2-4**) focuses on *genetic factors,* specifically on genetic polymorphisms in innate immunity receptors that affect acute rejection after LTx and susceptibility to infections in patients before and after LTx. In **Chapter 2** we examined the association between polymorphisms in TLR4 signaling pathway genes and severe bacterial infections in two independent cohorts of patients enlisted for LTx. In **Chapter 3** we investigated the association between genetic variants in MBL genes and the risk of infections in patients after LTx. In **Chapter 4** we determined the association between genetic variants in a broad range of innate immunity receptors and the risk of bacterial and fungal infections and acute rejection in two cohorts of patients post-LTx.

Part II (**Chapter 5-6**) describes the influence of *viral factors*, especially CMV, on T-cell alloresponses after LTx. In **Chapter 5** we compared distributions of circulating T-cell subsets and determined T-cell alloresponses in CMV-infected and non-infected LTx patients,

and in addition, the association between CMV and acute rejection after LTx. In **Chapter 6** we studied whether co-inhibitory receptors expressed on $CD8^+$ T cells affect allogeneic responses of these T cells. We also determined which factors induced the expression of co-inhibitory receptors that affect $CD8^+$ T-cell alloresponses, and found an important contribution of CMV infection.

Part III of the thesis (**Chapter 7-8**) describes strategies to optimize immunosuppression in patients after LTx, in order to optimize outcome in these patients. In **Chapter 7**, an overview is given of immunosuppressive drugs that are administered after LTx to prevent acute rejection. In this review, we describe clinical strategies to optimize immunosuppressive drug usage in patients after LTx, in order to avoid adverse effects of these drugs. **Chapter 8** highlights the immunosuppressive properties of liver graft-derived MSCs. We compare BM-MSCs and L-MSCs with regard to their suppressive capacity on T-cell alloresponses and describe how these findings in the future may lead to development of cell-based immunosuppressive therapies, as alternative for the currently used immunosuppressive drugs that involve serious side effects.

Finally, in part IV (**Chapter 9-10**), we summarize the findings of our studies on risk factors that are associated with the occurrence of complications in liver transplant patients and strategies to optimize the use of immunosuppression after LTx. We will address which questions have been answered, put them into perspective, and formulate new research questions to be pursued.

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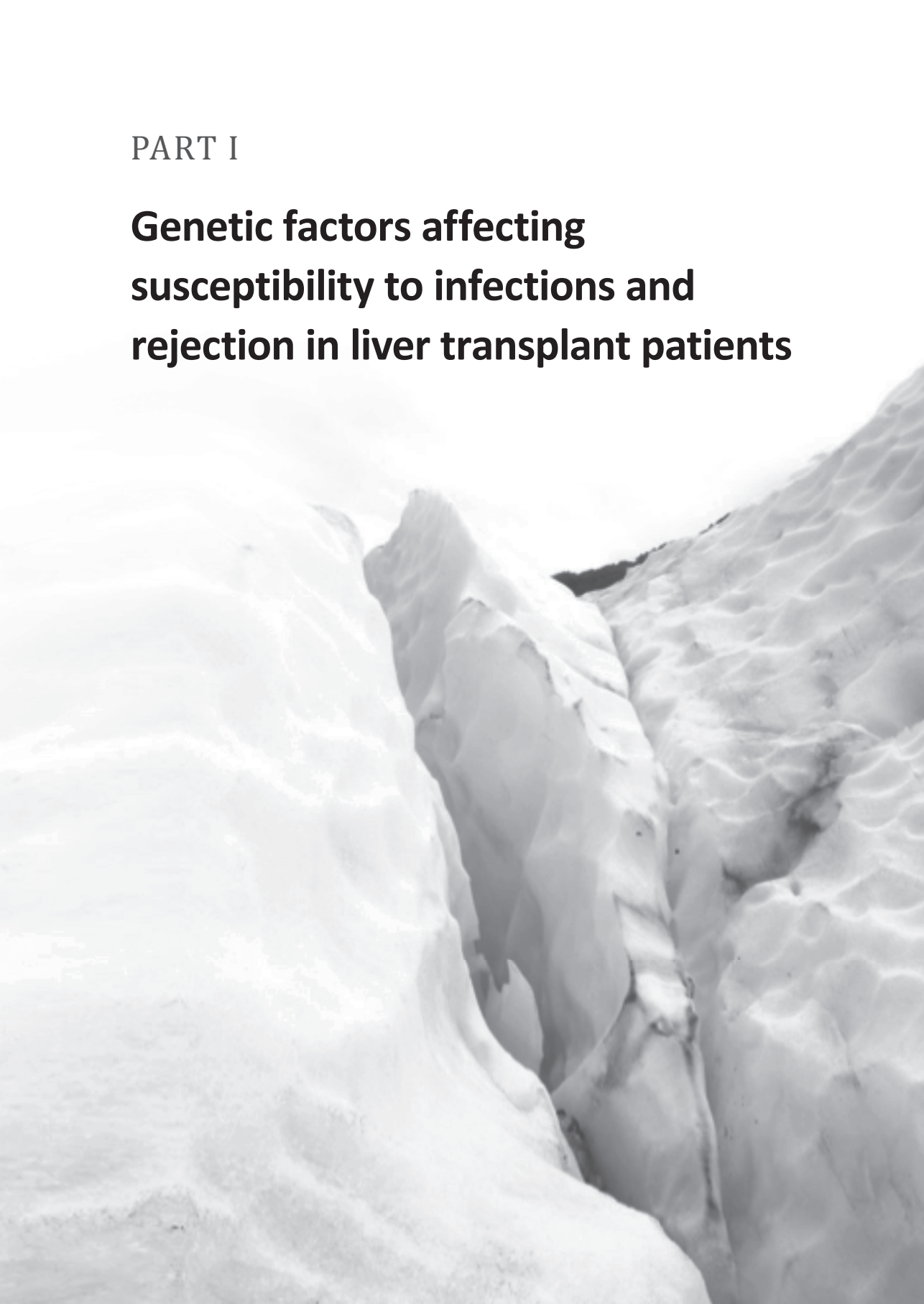
PART I

Genetic factors affecting susceptibility to infections and rejection in liver transplant patients



CHAPTER 2

Genetic variation in TNFA predicts protection from severe bacterial infection in patients with end-stage liver disease awaiting liver transplantation

Renata Senkerikova[1]*
Emmeloes de Mare-Bredemeijer[3]*
Sona Frankova[1]
Dave Roelen[4]
Thijmen Visseren[3]
Pavel Trunecka[1]
Julius Spicak[1]
Herold Metselaar[3]
Milan Jirsa[2]
Jaap Kwekkeboom[3]
Jan Sperl[1]

* shared first authorship
1 Department of Hepatogastroenterology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic
2 Laboratory of Experimental Hepatology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic
3 Department of Gastroenterology and Hepatology, Erasmus MC - University Medical Centre, Rotterdam, The Netherlands
4 Department of Immunohematology and Blood Transfusion, University Medical Centre, Leiden, The Netherlands

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ABSTRACT

Augmented susceptibility to infections increases mortality in patients with end-stage liver disease (ESLD). We sought to determine the contribution of selected genetic variants involved in inflammatory signalling downstream of the Toll-like receptor 4 (TLR4) to severe bacterial infections (SBIs) in patients with ESLD. We retrospectively assessed incidence of SBIs in 336 adult ESLD patients enlisted for orthotopic liver transplantation (OLT) and genotyped them for TLR4 c.+1196C/T, CD14 c.159C/T, TNFA c.238G/A, TNFA c.863C/A, IL1B c.31C/T and IL1RN variable number of tandem repeats allelic variants. Principal findings were validated in an independent cohort of 332 ESLD patients. Thirty-four percent of patients from the identification cohort and 40% of patients from the validation cohort presented with SBI while enlisted for OLT. The presence of the variant allele TNFA c.238A (rs361525) was associated with lower serum levels of TNF-α, and with significantly decreased risk of SBI in both cohorts. Multivariate analysis showed that the relative protection from SBI associated with this allele almost completely negated the increased susceptibility to SBI owed to advanced ESLD. Although not predictive of overall mortality, the presence of the TNFA c.238A allele was associated with a complete prevention of SBI-related pre-transplant deaths. Our results suggest that genetic variability in inflammatory signalling is associated with the development of SBI in patients with ESLD. Specifically, we identified the importance of the TNFA c.238A allele as a strong predictor of protection from SBI, and as a genetic marker associated with significantly improved pre-transplant survival in patients with SBI.

INTRODUCTION

Severe bacterial infections (SBIs) represent the second leading cause of death in patients with end-stage liver disease (ESLD) waitlisted for orthotopic liver transplantation (OLT) (1,2), with mortality approaching 10% in European registries (3) and 23% in the US (4). The high mortality attributed to SBIs in this patient category is driven by the impaired antimicrobial response associated with ESLD (5,6), and by progression of liver failure that is accelerated by severe infection (1,2). Cirrhotic patients are prone to develop SBIs because of compromised antimicrobial defence caused by liver synthetic failure, portal hypertension and bacterial translocation from the gut (1,2,5,7,8), in conjunction with altered function of immune cells, including impaired opsonizing and neutrophil phagocytic capacity (9,10). Therefore, early identification of patients with ESLD at risk for SBIs is of paramount importance, but indicators predicting the development of SBIs are missing. Toll-like receptors (TLRs) play a key role in innate immune responses by recognition of a broad range of microbial components and triggering signals critical for antimicrobial defences (11–13). Although substantially conserved across species, TLRs show a genetic variability that modulates their downstream signalling, potentially determining individual susceptibility to infections (14). It has been shown that the variant p.399Ile of TLR4 corresponding to the nucleotide substitution TLR4 c.+1196C/T (rs4986791) changes the ligand-binding site of the receptor (15) and, in one study with limited sample size, predisposed cirrhotic patients to infections (16). Plasma concentration of CD14 is affected by the promoter polymorphism c.159C/T (rs2569190) (17), which influences expression of the protein (18) and the risk of death in patients with sepsis (19). SNPs at the positions c.863 (rs1800630) and c.238 (rs361525) of the TNFA promoter independently influence transcription of this gene (20–22). The Interleukin 1 gene cluster on chromosome 2 contains genes IL1B and IL1RN encoding the pro-inflammatory IL-1β and the anti-inflammatory IL-1 receptor antagonist (IL-1RA) (23). The variant c.31T (rs1143627) in the IL1B promoter increases the transcriptional activity of this gene (24). The second intron of IL1RN contains a variable number of tandem repeats (VNTR) 86-bp long. Allele 2 (IL1RN2) increases the concentration of IL-1β *in vitro* (25) and increases mortality in septic patients (26). The above mentioned studies (16,19,26,27) demonstrated significant associations between genetic variants and susceptibility to bacterial infections. However, these studies were performed mainly on limited numbers of non-cirrhotic patients and were not validated. Therefore, we decided to evaluate the contribution of genetic variants in the TLR4 pathway to the development of SBI in large, well characterised independent cohorts of cirrhotic patients with ESLD enlisted for OLT in two centres.

PATIENTS AND METHODS

Patients and definition of severe bacterial infections

Identification cohort

Three hundred and thirty-six patients with liver cirrhosis with Child-Pugh class B and C were enrolled and retrospectively screened for the occurrence of one or more episodes of extrahepatic SBIs during their time on the waiting list and 270 days before enlistment to include also patients with a recent episode of SBI. These patients were sorted out of 708 adult cirrhotic patients who were enlisted for OLT in Prague between February 1995 and June 2010. Patients with Child-Pugh class A and patients with acute liver failure were excluded. Patients with Caroli disease and primary and secondary sclerosing cholangitis were excluded as well, since intrahepatic bacterial complications are characteristic for the natural course of these diseases. SBIs were defined as the following bacterial infections requiring hospitalization and treatment with intravenous antibiotics:

(a) Spontaneous bacterial peritonitis (SBP), diagnosis of which was based on neutrophil cell count exceeding 250/mm^3 and/or positive culture of ascitic fluid if secondary causes of peritonitis were excluded (EASL guidelines (28)).
(b) Urinary tract infections diagnosed on the basis of clinical findings (dysuria, fever), pyuria (leukocytes >10/mm^3) and positive urine culture (29).
(c) Pneumonia, diagnosis of which was determined by clinical symptoms (cough, expectoration, and fever), positive chest X-ray and positive bacteriological finding in sputum (30).
(d) Skin and soft tissue infection, diagnosis of which was established by local cutaneous findings (blush, tumefaction, and pain) and leukocytosis (31).
(e) Bacterial infection of unknown origin defined as a positive blood culture with serum C-reactive protein (CRP) level ≥70 mg/L.

All clinical data were collected from hospitalisation and outpatient medical records archived at our centre.

Validation cohort

The validation cohort (n = 332 cirrhotic patients enlisted for OLT) was selected from the 522 adult patients evaluated for OLT between September 1995 and April 2011 in Erasmus MC-University Medical Centre, Rotterdam, The Netherlands. The selection process was based on the same criteria as in the identification cohort. In 332 selected patients, SBIs were defined according to the same definitions as used in the identification cohort from Prague. A higher rate of Child-Pugh B patients was observed in the validation cohort be-

cause of the Dutch policy to enlist patients for OLT when they had Child-Pugh score 8 (B) or higher. Another reason to enlist patients with cirrhosis staged as Child-Pugh B was the presence of hepatocellular carcinoma. Due to progression of liver disease on the waiting list, most patients had Child-Pugh score C at the moment they were transplanted.

Genotyping

Patients were genotyped for TLR4 c.+1196C/T, CD14 c._159C/T, TNFA c._238G/A, TNFA c._863C/A, IL1B c._31C/T and IL1RN VNTR (UniSTS:156109) allelic variants, as described in (32), using specific primers and PCR conditions shown in Supplementary Table 1. In order to minimise genotyping errors, blank control wells were left on the PCR plates and two operators, unaware of the status of the samples, performed the genotype assignment independently. After testing for Hardy-Weinberg equilibrium (HWE), allele frequencies were checked for consistency with data from the population of European ancestry (Utah Residents with Northern and Western European Ancestry) from the HapMap database (33).

Primary assessment of associations between allelic frequencies and SBIs was performed in the identification cohort from Prague, and positive associations were confirmed in the validation cohort from Rotterdam. The study was approved by the institutional Research Ethics Committee of both participating centres. Written informed consent with DNA sampling was obtained from all patients and the study conformed to the declaration of Helsinki EthicalGuidelines.

Determination of serum levels of TNF-α

Serum levels of TNF-α were determined in blood samples taken from patients at the moment of liver transplantation, i.e., in patients with no physical and laboratory signs of infection. The samples were frozen immediately after serum separation and stored at -80°C. In the identification cohort, TNF-α was assessed in serum samples of 199 patients, out of which 179 patients were homozygotes for the TNFA c._238G allele and 20 patients were heterozygotes. Additional 36 samples (12 samples of patients carrying the TNFA c._238GA genotype and 24 samples of homozygotes for TNFA c._238G) came from the validation cohort. Quantitative determination of TNF- α was performed with the Quantikine HS ELISA human TNF-α immunoassay (R&D Systems, Abingdon, UK). All standards, controls and samples were analysed in duplicates and the duplicate readings were averaged. Duplicates with coefficient variability (CV) higher than 50% (5 heterozygotes and 25 homozygotes) were excluded and the remaining 27 heterozygotes were then matched in age, sex, and underlying diseases with 81 of the 188 non-excluded heterozygotes.

Statistical analysis

Data are presented as mean and standard deviation, as median and range, or as frequencies, as appropriate. HWE and differences in genotype frequencies between patients with SBI and controls were analysed using two-sided χ^2 testing. Using standard formulas based on two-by-two tables (34), we calculated basic epidemiology statistics and evaluated the preventable fractions among the population and among the exposed. *t* tests or Mann-Whitney tests were used for comparisons of the means. Due to the testing of multiple statistical hypotheses, Bonferroni correction was used in the identification cohort. Cox regression analysis was used to calculate hazard ratio and 95% confidence interval (CI). Significant risk factors from univariate analysis were entered into the multivariate Cox regression analysis, which was performed with a forward stepwise approach. Wald statistics was employed in the regression module to evaluate the relative contribution of significant variables to SBI. Kaplan-Meier analysis with log-rank test was performed to evaluate survival. A p value <0.05 was considered statistically significant throughout the study. Statistical analysis was performed using JMP 9.0.0. and SPSS 13.0 programs.

RESULTS

Demographic, clinical and survival data

Demographic and clinical data of OLT candidates included in the study are shown in Table 1. The median time to develop SBI while on the waiting list, or the median time to OLT in patients who did not develop SBI, was shorter in the identification cohort compared to the validation cohort (337 vs. 479 days, p <0.001) and a higher proportion of patients with Child-Pugh B classification were present in the validation cohort (59.9% vs. 50.6%, p = 0.015). Both cohorts contained similar spectra of liver diseases (Table 1). The group of cholestatic liver diseases included primary biliary cirrhosis, secondary biliary cirrhosis, PBC/AIH overlap syndrome and benign recurrent intrahepatic cholestasis/Byler's disease. Metabolic liver diseases group comprised Wilson's disease, α1-antitrypsin deficiency, haemochromatosis, and erythropoietic protoporphyria, and liver cirrhosis of other aetiologies involved cryptogenic cirrhosis, Budd-Chiari syndrome and non-alcoholic steatohepatitis. Significantly more patients with liver cirrhosis due to chronic hepatitis B were present in the validation cohort compared with the identification cohort (15.4% vs. 8.6%). Out of all patients evaluated for OLT, 32.1% patients in the identification cohort and 51.5% in the validation cohort died; 10.7% and 28.6% of patients died pre-transplant at a median of 84 and 206 days after enlistment for OLT, and 21.4% and 22.9% of patients died at a median of 1109 and 969 days after OLT, respectively (Table 1, bottom sections). Approximately 42% pretransplant and 31% post-transplant deaths were attributable to

SBI in the identification cohort. In the validation cohort, SBI were responsible for 38% deaths in the pre-transplant period and for 28% deaths post-transplant.

Table 1. Demographic, clinical and survival data of patients listed for OLT

	Identification cohort	Validation cohort	P value
N	336	332	
Age at OLT (years, mean ± SD)	53 ± 9.5	52 ± 10.2	NS
Male/Female (%)	194/142 (57.7/42.3)	216/116 (65.1/34.9)	NS
Interval between enlistment and SBI or OLT (days, median - range)	337 (270-1335)	479 (272-2979)	<0.001
Survival after enlistment (days, median - range)	2770 (271-6079)	2994 (274-6834)	NS
Child-Pugh B/C (%)	170/166 (50.6/49.4)	199/133 (59.9/40.1)	0.015
MELD (points, median - range)	15.8 (6-40)	15.6 (1-40)	NS
Aetiology of cirrhosis	**N (%)**	**N (%)**	***P* value**
ALD	119 (35.4)	97 (29.2)	NS
HCV	65 (19.3)	70 (21.1)	NS
HBV	29 (8.6)	51 (15.4)	0.003
Cholestatic	49 (14.6)	29 (8.7)	NS
Metabolic	15 (4.5)	13 (3.9)	NS
AIH	23 (6.8)	17 (5.1)	NS
Other	36 (10.7)	55 (16.6)	NS
Mortality	**N (%)**	**N (%)**	***P* value**
No death	228 (67.9)	161 (48.5)	<0.001
Death prior to OLT	36 (10.7)	95 (28.6)	
Death after OLT	72 (21.4)	76 (22.9)	
Pre-transplant deaths			
Survival (median days, IQR)	84 (38-200)	206 (37-477)	0.002
SBI-related deaths (N, %)	15 (41.7)	36 (37.9)	NS
SBI-unrelated deaths (N, %)*	21 (58.3)	59 (62.1)	
Post-transplant deaths			
Survival (median days, IQR)	1109 (200-2322)	969 (68-2073)	NS
SBI-related deaths(N, %)	22 (30.6)	28 (36.8)	NS
SBI-unrelated deaths (N, %)**	50 (69.4)	48 (63.2)	

Child-Pugh and MELD score at the time of enlistment for liver transplantation; ALD – alcoholic liver disease; HCV – hepatitis C virus; HBV – hepatitis B virus; AIH – autoimmune hepatitis. IQR – interquartile range; NS – not significant

*) causes of SBI-unrelated pre-transplant deaths: Liver failure 9 (42.9%); cardiovascular 7 (33.3%); GIT bleeding related to portal hypertension 3 (14.3%); other 2 (9.5%) in identification cohort and liver failure 24 (40.7%); cardiovascular 4 (6.8%); GIT bleeding related to portal hypertension 6 (10.2%); other and unknown 25 (42.4%) in validation cohort

**) causes of SBI-unrelated post-transplant deaths: Malignancy 22 (44%); cardiovascular 19 (38%); graft failure 7 (14%); other 2 (4%) in identification cohort and malignancy 10 (20.8%); cardiovascular 15 (31.3%); graft failure 18 (37.5%); other 5 (10.4%) in validation cohort

Severe bacterial infections

Thirty-four percent of patients with advanced liver cirrhosis in the identification cohort and 40% of patients in the validation cohort presented with SBI during the observation period (Table 2). In the identification cohort, the risk of SBI was significantly associated with female gender and the degree of liver dysfunction evaluated by MELD score or Child-Pugh score. Both MELD score and Child-Pugh score but not female gender remained significantly associated with SBI in the validation cohort. Patients in the validation cohort suffering from SBI had a significantly shorter observation period than patients without SBI (409 vs. 499 days, p = 0.037). Age and aetiology of liver cirrhosis were not associated with susceptibility to SBI (Table 2). A total of 130 episodes of SBI were diagnosed in 115 patients in the identification cohort compared with 170 episodes of SBI diagnosed in 133 patients in the validation cohort. The frequencies of most SBIs were similar in identification and validation cohorts: pneumonia 10/130 vs. 20/170, p = 0.244, urinary tract infection 21/130 vs. 25/170, p = 0.730, and infection of skin and soft tissues 8/130 vs. 10/170, p = 0.922. However, SBP was significantly more frequent in the identification cohort (84/130 vs. 90/170, p = 0.042) whereas patients with bacterial infections of unknown origin were more prevalent in the validation cohort (7/130 vs. 25/170, p = 0.001).

Table 2. Demographic and clinical data of patients with and without severe bacterial infection

	Identification cohort			**Validation cohort**		
	with SBI	**without SBI**	**P value**	**with SBI**	**without SBI**	**P value**
N (%)	115 (34.2)	221 (65.8)		133 (40.1)	199 (59.9)	
Age at OLT (years, mean ± SD)	52 ± 10.6	53 ± 8.9	NS	52 ± 10.4	52 ± 10.1	NS
Male/Female (%)	53/62 (46.1/53.9)	141/80 (63.8/36.2)	0.002	84/49 (63.2/36.8)	132/67 (66.3/33.7)	NS
Interval between enlistment and SBI or OLT (days, median - range)	333 (271-1335)	338 (270-709)	NS	409 (274-2979)	499 (272-2644)	0.037
Child-Pugh B/C (%)	46/69 (40/60)	124/97 (56.1/43.9)	0.005	60/73 (45.1/54.9)	139/60 (69.8/30.2)	<0.001
MELD (points, median - range)	16 (6-36)	15 (7-40)	0.004	17 (7-40)	14 (1-34)	<0.001
Aetiology of cirrhosis	**N (%)**	**N (%)**	***P* value**	**N (%)**	**N (%)**	***P* value**
ALD	41 (35.7)	78 (35.3)	NS	46 (34.6)	51 (25.6)	NS
HCV	21 (18.3)	44 (19.9)	NS	23 (17.3)	47 (23.6)	NS
HBV	8 (7.0)	21 (9.5)	NS	25 (18.8)	26 (13.1)	NS
Cholestatic	16 (13.9)	33 (14.9)	NS	10 (7.5)	19 (9.6)	NS
Metabolic	5 (4.3)	10 (4.5)	NS	6 (4.5)	7 (3.5)	NS
AIH	9 (7.8)	14 (6.3)	NS	6 (4.5)	11 (5.5)	NS
Other	15 (13.0)	21 (9.5)	NS	17 (12.8)	38 (19.1)	NS

Child-Pugh and MELD score at the time of enlistment for liver transplantation; ALD – alcoholic liver disease; HCV – hepatitis C virus; HBV – hepatitis B virus; AIH – autoimmune hepatitis. NS – not significant

Genetic associations with severe bacterial infections

The identification cohort was genotyped for the 6 annotated SNP loci. Genotype frequencies at the individual loci were in HWE except for the TNFA c._863C/A locus, which was excluded from further evaluations. Single locus analysis performed in the identification cohort (n = 336, Table 3 and Fig. 1) revealed a strong association of the TNFA c._238 locus with SBIs. Subjects carrying the minor TNFA c._238A allele showed significantly reduced risk of SBI (OR 0.22, 95% CI 0.07–0.75, $p_{non\text{-}adjusted}$ = 0.008, $p_{adjusted}$ (Bonferroni) = 0.042) compared with homozygotes for the major TNFA allele c._238G. Among the genetic loci under study, only this allele was associated with SBI; neither of the other investigated genes showed any differences in the distribution of genotype frequencies between cases and controls (Table 3 and Fig. 1). Similar to the identification cohort, presence of the TNFA c._238A allele conferred a significantly decreased risk of SBI in the validation cohort (OR 0.37, 95% CI 0.14–1.02, p = 0.046, Table 3) and the association was independent of aetiol-

Table 3. Genotype distributions in the identification and validation cohort

		Identification cohort			Validation cohort		
		Patients with SBI (n=115)	Patients without SBI (n=221)	P	Patients with SBI (n=133)	Patients without SBI (n=199)	P
Locus	Genotype	N (%)	N (%)		N (%)	N (%)	
CD14 c.-159	CC	32 (27.8)	62 (28.1)	NS			
	CT	62 (53.9)	111 (50.2)				
	TT	21 (18.3)	48 (21.7)				
TLR4 c.1196	CC	101 (87.8)	196 (88.7)	NS			
	CT	14 (12.2)	25 (11.3)				
	TT	0	0				
TNFA c.-238	GG	112 (97.4)	197 (89.1)	0.008	128 (96.2)	180 (90.5)	0.046
	GA	3 (2.6)	24 (10.9)		5 (3.8)	19 (9.5)	
	AA	0	0		0	0	
TNFA c.-863	CC	91 (79.1)	184 (83.3)	n/a*			
	CA	18 (15.7)	26 (11.8)				
	AA	6 (5.2)	11 (5.0)				
IL1B c.-31	CC	13 (11.3)	33 (14.9)	NS			
	CT	58 (50.4)	103 (46.6)				
	TT	44 (38.3)	85 (38.5)				
IL1RN VNTR	11	47 (40.9)	103 (46.6)	NS			
	12	51 (44.3)	91 (41.2)				
	22	13 (11.3)	14 (6.3)				
	14, 31, 32	4 (3.5)	13 (5.9)				

*) As TNFA c.-863 genotypes were not in HWE, no further calculations were performed. NS – not significant

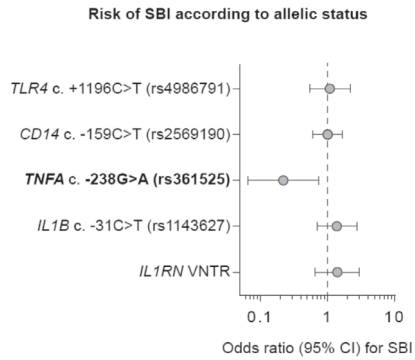

Figure 1.
Association of the investigated variants with SBI in the identification cohort. Bars represent OR with 95% confidence interval. Source data are shown in Table 3.

ogy of SBI in both cohorts (data not shown). The presence of minor TNFA c._238A allele has been previously shown to decrease the transcriptional activity of TNFA resulting in lower production of TNF-α protein (21). Consistent with this report, we found that 27 carriers of the variant TNFA c._238A allele showed significantly lower levels of serum TNF-α, compared with 81 age-, sex- and diagnosis-matched homozygotes for the major allele TNFA c._238G (2.13 ± 2.11 pg/ml vs. 3.11 ± 2.52 pg/ml, p = 0.022) (Fig. 2). The difference between the 27 heterozygotes and all 188 homozygotes was even more pronounced (2.13 ± 2.11 pg/ml vs. 3.52 ± 3.27 pg/ml, p = 0.006).

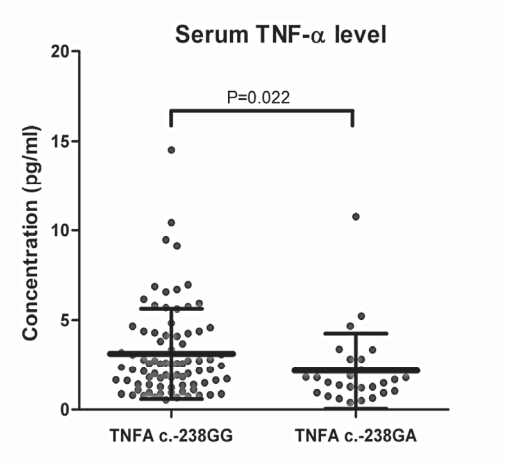

Figure 2.
Decreased serum levels of TNF-α in carriers of the TNFA c._238A variant. The data from 81 homozygotes (c._238GG) and 27 heterozygotes (c._238GA) are shown as individual dots. Horizontal bars indicate mean (thick line) and standard deviations (thin lines). Mann-Whitney was used for comparison of the means.

Determinants of severe bacterial infection and mortality

The potential role of TNFA c._238A as an independent predictor of SBI was evaluated by Cox regression analysis. Due to the low frequency of the TNFA c._238A allele (about 4% in both groups), we pooled both cohorts to achieve a sufficient power to detect moderate and small effect sizes. Calculations of hazard ratios in the univariate mode showed that SBIs were significantly associated with MELD, Child-Pugh class C vs. B) and TNFA c._238

Table 4. Risk factors of SBI or death

I. Outcome: Severe bacterial infection				
A. Univariate analysis				
Variables	**Wald**	**HR**	**95% CI**	**P value**
MELD per 1 point increment	21.918	1.072	1.041-1.104	<0.001
Child-Pugh class C *vs* B	6.89	1.550	1.117-2.150	0.009
TNFA c.-238A	4.869	0.427	0.200-0.914	0.028
Gender (female *vs* male)	2.879	1.293	0.961-1.741	0.09
Cohort (identification *vs* validation cohort)	0.803	1.149	0.448-1.557	NS
Age per 1 year increment	0.580	1.006	0.991-1.020	NS
B. Multivariate analysis				
Variables	**Wald**	**HR**	**95% CI**	***P* value**
MELD per 1 point increment	19.964	1.067	1.037-1.098	<0.001
Child-Pugh class C *vs* B	6.61	1.531	1.107-2.119	0.01
TNFA c.-238A	5.044	0.421	0.197-0.896	0.025
II. Outcome: Pre-transplant death (all causes)				
A. Univariate analysis				
Variables	**Wald**	**HR**	**95% CI**	***P* value**
SBI	32.343	2.36	1.755-3.173	<0.001
Age per 1 year increment	17.609	1.033	1.018-1.049	<0.001
MELD per 1 point increment	11.431	1.056	1.023-1.09	<0.001
Gender (female *vs* male)	10.647	0.606	0.448-0.819	0.001
Child-Pugh class C *vs* B	0.972	1.169	0.857-1.595	NS
Cohort (identification *vs* validation cohort)	0.959	1.161	0.861-1.565	NS
TNFA c.-238A	0.004	1.017	0.606-1.707	NS
B. Multivariate analysis				
Variables	**Wald**	**HR**	**95% CI**	***P* value**
SBI	38.58	2.476	1.860-3.296	<0.001
Age per 1 year increment	17.509	1.033	1.017-1.049	<0.001
MELD per 1 point increment	16.259	1.062	1.031-1.093	<0.001
Gender (female *vs* male)	14.296	0.572	0.428-0.764	<0.001

HR, hazard ratio; CI, confidence interval; Wald, measurement of influence statistics
Analyses were performed in pooled patient cohorts (n=668) using Cox regression. NS – not significant

status, but not with age and gender. Multivariate analysis confirmed that MELD, Child-Pugh score and the TNFA c._238 genotype represent independent predictors of SBIs (Table 4). Specifically, advanced liver disease (Child-Pugh class C vs. B) increased the risk of SBI 1.5-fold and each one-point increment in MELD score increased the risk of SBI by approximately 7%, whereas presence of the variant TNFA c._238A allele decreased risk of SBI by almost 2.5-fold (Table 4, multivariate analysis). Using Wald statistics to evaluate the relative contributions of these determinants to SBI, we found that the presence of the variant TNFA c._238A allele mitigated the odds of SBI developing as a consequence of Child-Pugh C class liver dysfunction by 76% (5.044/6.61) (Table 4, part I.B). Further calculations demonstrated that presence of the minor TNFA c._238A allele prevented 7.2% (95% CI 3.7–8.8%) of SBIs in the whole cohort (preventable fraction among the population), and that among the patients who developed SBIs, 70.6% (95% CI 36.7–86.4%) of these cases would hypothetically be preventable by the presence of the minor TNFA c._238A allele (preventable fraction among the exposed). The presence of TNFA c._238A

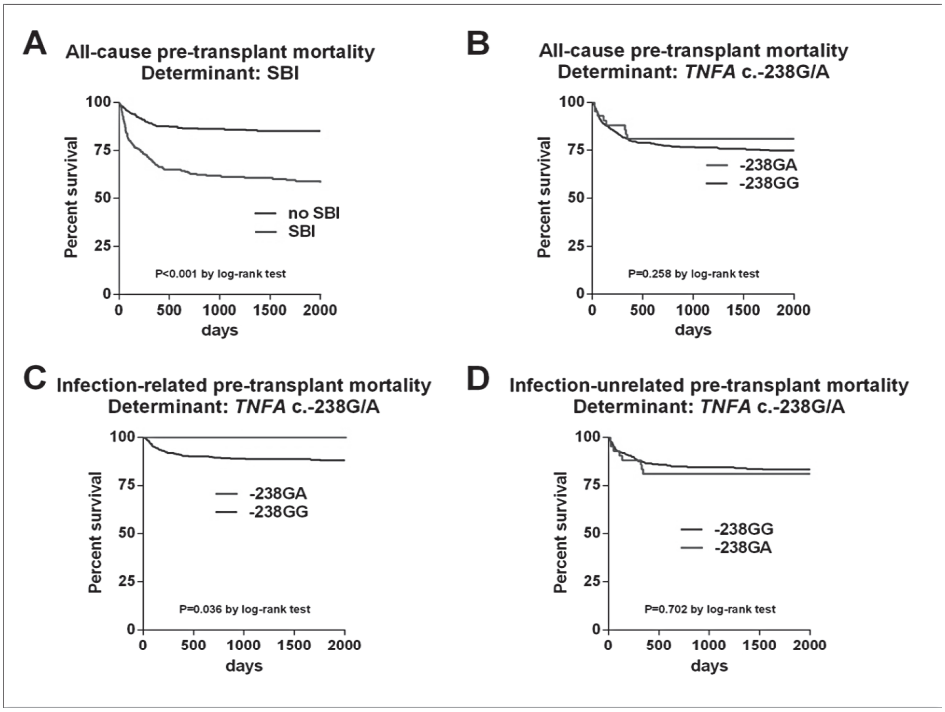

Figure 3.
The presence of the TNFA c._238A variant is associated with significantly decreased risk of SBI-related pre-transplant mortality. The impact of SBI (A) and TNFA c._238GA genotype (B) on overall pre-transplant mortality and the association of TNFA c._238GA genotype with mortality attributable to SBI (C) or non-infectious causes (D) were analyzed in pooled patient cohorts (n = 668). Kaplan-Meier analysis with Log-rank test was used for statistical evaluation.

predicted protection from SBIs in the pooled cohort with a high positive predictive value (84.4%; 95% CI 72.4–98.0%) and specificity (89.8%; 95% CI 86.0–92.5%), whereas sensitivity and negative predictive value were low (9.3% and 38.9%, respectively). Collectively, these findings demonstrate that possession of the variant TNFA c._238A allele provides strong protection against SBI in patients with advanced end-stage liver disease. Finally, we evaluated TNFA c._238A allele as a predictor of mortality. Multivariate Cox regression analysis identified SBI, age, female gender and MELD score as significant predictors of overall pre-transplant mortality (Table 4, part II and Fig. 3A). In contrast, the TNFA c._238 genotype was associated with all-cause mortality neither in regression modelling (Table 4, part II), nor in Kaplan-Meier analysis (Fig. 3B). However, as our previous data demonstrated significant association of TNFA c._238 genotype with SBI (Table 3, Fig. 1), and SBI was strongly associated with mortality (Table 4, part II and Fig. 3A), we hypothesized that TNFA c._238 genotype could be a conditional predictor of mortality based on infection status of the host. Therefore, we performed a subgroup analysis and found that patients carrying the TNFA c._238A allele had a significantly decreased risk of succumbing to SBI compared with homozygotes for the major GG genotype (Fig. 3C). Specifically, none of the carriers of the TNFA c._238A allele who developed SBI in the pre-transplant period died, whereas SBI-related mortality in homozygotes for the major allele TNFA c._238G reached 12% (Fig. 3C). As expected, there was no association between TNFA c._238 and pre-transplant death from non-infectious causes (Fig. 3D).

DISCUSSION

Our study provides several lines of evidence suggesting that the TNFA c._238 status is a significant predictor of reduced susceptibility to SBIs. First, the variant allele was associated with SBIs in two independent cohorts of patients with ESLD. Second, its association with SBI was independent of other variables in multivariate Cox regression analysis. Third, the relative significance of the TNFA c._238 allelic status in predicting SBIs was in close succession to MELD score and Child-Pugh C class of liver dysfunction, the strongest determinants of SBIs identified in this study. Given the low prevalence of the TNFA c._238A allele, the potential clinical utility of our finding would be in ruling out the potential risk of SBIs in a minor group of patients with ESLD. Considering that the TNFA c._238A allele is a marker of protection from SBIs, the presence of this allele in any given individual with ESLD will predict significant protection from SBIs (84% positive predictive value for the protection from SBIs in carriers of the variant A allele). Moreover, presence of this allele in patients with SBI awaiting OLT predicts a substantial survival benefit. The findings are consistent with the relevant contribution of the TNFA c._238 allelic status to the risk of SBI in multivariate Cox regression analysis and indicate that

the presence of the TNFA c._238A allele may be truly significant in the clinical scenario. However, although the allelic status aids in ruling out SBI, it has a very low ruling-in characteristic, since the major allele G is abundantly represented in both SBI and non-SBI groups. Although several allelic variants were investigated in this study, only the TNFA c._238 allele, but not variants in TLR4, CD14, IL1B, and IL1RN, were associated with SBIs. This is consistent with the hierarchical pattern of TLR signaling. Although TLR2, 4, 5, and 9 recognize distinct microbial components (peptidoglycan from G+ bacteria, LPS from G- bacteria, flagellin from G+ and G- bacteria, and unmethylated CpG sequences in bacterial DNA, respectively), they all utilize the common adaptor MyD88 and the nuclear factor kappa-B to induce inflammatory cytokines TNF-α and IL-1β (13). Therefore, as SBIs are caused by a wide spectrum of pathogens (1,35), it is possible that the major signal for induction of MyD88-dependent inflammatory cytokines in SBIs comes from a source other than LPS, which could explain the lack of association of TLR4 and CD14 with SBIs in our study. This notion is further supported by our data that multiple microbial classes determined SBIs in our study.

The lack of association between the IL1B gene cluster and SBIs could be attributed to differential roles of TNF-α vs. IL-1β in immune responses. Whereas TNF-α is triggered by bacterial ligands and is indispensable for antimicrobial defence, IL-1β serves as an amplifier of inflammatory responses and requires co-activation by host-derived factors released from tissues upon damage (36), which may not always be present in the context of bacterial infection. The TNFA c._238A allele determines low transcriptional activity of the TNFA gene and lower production of TNF-α *in vitro* (21,22) as well as *in vivo* (our study, Fig. 2). This seemingly paradoxical finding is consistent with desensitisation of innate immune cells to bacterial components and significant neutrophilic dysfunction found in patients with liver cirrhosis. Tritto et al. (9) reported that severity of liver disease positively correlates with serum levels of TNF-α and negatively correlates with neutrophil phagocytic dysfunction. This suggests that chronic elevation of TNF-α level causes exhaustion of neutrophil opsonizing and phagocytic capacity (9). The hypothesis is further supported by Ono et al. (10), who found desensitisation of neutrophils to TNF-α and LPS stimulation resulting in opsonophagocytic dysfunction in patients with liver cirrhosis. Finally, Stadlbauer et al. (37) demonstrated the neutrophil phagocytic dysfunction in patients with alcoholic cirrhosis was restored by treatment with probiotics, which resulted in decreased endotoxemia and TNF-α production. Our finding that the low-producer genotype in the TNFA c._238 locus negatively affects the risk of SBIs is therefore fully consistent with the aforementioned studies; it furthermore supports the biological concept of immune cell desensitization by overzealous activation of inflammatory signalling in patients with liver cirrhosis, and implies that the low-producing variant TNFA c._238A allele in patients with ESLD may reset the sensitivity of innate immune cells to microbial

ligands. The concept of neutrophil exhaustion is also strongly supported by their lower serum levels of TNF-α. Low TNF-α production might have unfavourable consequences in cirrhotic patients manifested as higher occurrence of hepatocellular carcinoma as described by Teixeira et al. (38). This observation might be explained by deficient anti-tumour immunity in individuals with decreased levels of TNF-α. Nonetheless, the beneficial effect consisting of lower incidence of SBI and significantly decreased mortality owing to SBIs in patients awaiting OLT predominates over the risk of tumour growth. The main limitation of our study resides in its retrospective design. For this reason we were not able to validate the published data (21,22) on the decreased transcriptional activity of TNFA in heterozygotes for the c._238A allele. On the other hand, we assessed serum levels of TNF-α in a representative subset of enrolled patients and provided indirect evidence supporting such hypothesis.

In conclusion, we have shown that presence of the TNFA c._238A allele in ESLD patients virtually excludes the probability of developing SBIs or dying of SBI in patients awaiting OLT. The strong effect of this allele supports the biological concept that TNF-mediated desensitisation of innate immune cells drives increased susceptibility to bacterial infections in patients with ESLD. Our findings indicate potential utility of TNFA rs361525 genotyping in assessment of individual risk of SBIs in patients waitlisted for OLT and warrant further mechanistic studies.

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Supplementary table. Genotyped loci in CD14, TLR4, TNFA, IL1B and IL1RN

Variation Ref. SNP[a] ID	Forward primer Reverse primer	T_m, °C Time [s]	Restriction enzyme Fragment length (bp)
CD14	5'-TTGGTGCCAACAGATGAGGTTCAC-3'	60°C	*Hae*III
c.-159C/T	5'-TTCTTTCCTACACAGCGGCACCC-3'	30 s	204, 201, 156
rs2569190			
TLR4 c.+1196C/T	5'-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3'	55°C	*Hinf*I
rs4986791	5'-ACCTGAAGACTGGAGAGTGAGTTAAATGCT-3'	40 s	378, 29
TNFA c.-238G/A	5'-GCCCCTCCCAGTTCTAGTTC-3'	62°C	*Bam*HI
rs361525	5'-CTCACACTCCCCATCCTCCCGGATC-3'	30 s	185, 26
TNFA c.-863C/A	5'- GGCTCTGAGGAATGGGTTAC-3'	63°C	*Tai*I
rs1800630	5'- CTACATGGCCCTGTCTTCGTTACG-3'	30s	125, 104, 21
IL1B	5'-CCCTTCCATGAACCAGAGAA-3'	60°C	*Alu*I
c.-31C/T	5'-GCTGAAGAGAATCCCAGAGC-3'	30 s	97, 87, 54
rs1143627			
IL1RN VNTR	5'-CCCCTCAGCAACACTCC-3'	64°C	-
156109[b]	5'-GGTCAGAAGGGCAGAGA-3'	30 s	442, 270, 528, 356, 614

[a]SNP database of genetic variation, www.ncbi.nlm.nih.gov/SNP. [b]STS database of unified sequence tagged sites, www.ncbi.nih.gov/genome/STS, VNTR, variable number of tandem repeats; Tm, melting temperature.

CHAPTER 3

Does the donor mannose-binding lectin genotype really predict the risk of bacterial infections after liver transplantation?

Lilian A. Curvelo[1]
Emmeloes de Mare-Bredemeijer[1]
Ilse de Canck[3]
Martine van Thielen[3]
Geert Kazemier[2]
Herold Metselaar[1]
Jaap Kwekkeboom[1]

1 Department of Gastroenterology and Hepatology, Erasmus MC - University Medical Center Rotterdam, Rotterdam, the Netherlands
2 Department of Surgery, Erasmus MC - University Medical Center Rotterdam, Rotterdam, the Netherlands
3 R&D Discovery, Innogenetics NV, Zwijnaarde, Belgium

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Based on letter to the editor.

With great interest, we read the recent article by De Rooij et al (1) and the accompanying editorial (2). The authors showed that functional single-nucleotide polymorphisms within donor genes involved in the lectin complement pathway [mannose-binding lectin 2 (MBL2), ficolin 2, and mannan-binding lectin-associated serine protease 2 (MASP2)] determine the risk of bacterial infections after liver transplantation (LT). Although this is the first study associating single-nucleotide polymorphisms in ficolin 2 and MASP2 with the risk of infection after LT, the value of the donor MBL2 genotype as a risk factor for infection after LT is supported by two other studies (3,4). However, a fourth study (5) found no difference in the overall rate of infections between patients who received liver transplants from donors with insufficient MBL genotypes and those who received liver transplants from donors with sufficient MBL genotypes; although there was a higher incidence of septic shock after transplantation with MBL-insufficient livers. Moreover, the published studies used different ways to stratify MBL genotypes into groups with MBL serum levels predicted to be sufficient or insufficient. Donor YA/YA and YA/XA genotypes result in high serum MBL2 levels, O/O and XA/O genotypes are almost MBL2-deficient, and YA/O and XA/XA genotypes are associated with intermediate MBL2 serum levels after LT (4). Although De Rooij et al. used a strict definition of MBL insufficiency and considered only O/O and XA/O genotypes to be MBL-insufficient, Worthley et al. (4) also considered the intermediate XA/XA genotype to be MBL-insufficient, and the two other studies (3,5) also included the second intermediate genotype (YA/O) in the MBL-insufficient group. Therefore, the value of the donor MBL genotype and the method of its interpretation for the early identification of LT patients at risk of infectious complications are not established yet.

We studied the predictive value of donor MBL genotyping for bacterial infections in our own center. The MBL genotypes of 290 donor livers used for orthotopic transplantation between 1987 and 2010 were determined. Notably, this cohort represents the largest single-center cohort of LT patients in which associations between the donor MBL2 genotype and bacterial infections have been analyzed. In three different ways, we categorized donor livers as MBL-sufficient or MBL-insufficient according to the stratification systems used in the cited studies, and we analyzed associations with clinically significant and laboratory-confirmed bacterial infections occurring during the first 3 months after LT by chi-square analysis with Fisher's exact test (Table 1). Thirty-eight percent of LT recipients experienced one or more infectious episodes, and this is comparable to the numbers reported by the previous studies (1,4,5). Importantly, none of the three stratifications resulted in a statistically significant association between the donor MBL genotype and clinically significant infections. In addition, when we analyzed associations with site-specific infections, independently of MBL genotype stratification, we observed no significant increases in the risk of intra-abdominal infections or bacteremia in patients

who underwent transplantation with MBL-insufficient livers. However, in two of the three types of MBL genotype stratification, significantly more pneumonia was diagnosed in patients who underwent transplantation with MBL-deficient livers.

In conclusion, this retrospective study indicates that in our center, the donor MBL2 genotype is not helpful in predicting the risk of bacterial infection after LT.

Table 1. Associations between donor MBL2 genotypes and clinically significant bacterial infections after LTx

MBL-genotype stratification				
I[a]	**CSI**	**Intra-abdominal**	**Bacteremia**	**Pneumonia**
Insufficient: O/O, XA/O	22/47 (47%)	15/47 (32%)	12/47 (26%)	6/47 (13%)
Sufficient: YA/YA, YA/XA, XA/XA, YA/O	88/243 (36%)	58/243 (24%)	47/243 (19%)	12/243 (5%)
p-value	0.190	0.272	0.328	0.090
II[b]				
Insufficient: O/O, XA/O, XA/XA	31/66 (47%)	17/66 (26%)	18/66 (27%)	9/66 (14%)
Sufficient: YA/YA, YA/XA, YA/O	79/224 (35%)	56/224 (25%)	41/224 (18%)	9/224 (4%)
p-value	0.112	0.873	0.120	0.008
III[c]				
Insufficient: O/O, XA/O, XA/XA, YA/O	51/131 (39%)	28/131 (21%)	30/131 (23%)	13/131 (10%)
Sufficient: YA/YA, YA/XA	59/159 (37%)	45/159 (28%)	29/159 (18%)	5/159 (3%)
p-value	0.808	0.221	0.380	0.026

[a]MBL-genotypes stratified according to the combinations of promoter -221 and exon 1 SNPs used by De Rooij et al (1). [b]MBL-genotypes stratified according to Worthley et al (4). [c]MBL-genotypes stratified according to Bouwman et al (3) and Cervera et al (5).

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CHAPTER 4

Genetic polymorphisms in innate immunity receptors do not predict the risk of bacterial and fungal infections and acute rejection after liver transplantation

E.L.D. de Mare-Bredemeijer[1]
S. Mancham[1]
W.K. Utomo[1]
I. de Canck[2]
M. van Thielen[2]
E. de Meester[2]
R. Rossau[2]
L.J.W. van der Laan[3]
B.E. Hansen[1,4]
H.W. Tilanus[3]
G. Kazemier[3]
H.L.A. Janssen[1]
H.J. Metselaar[1]
J. Kwekkeboom[1]

1 Department of Gastroenterology and Hepatology, Erasmus MC – University Medical Center Rotterdam, Rotterdam, The Netherlands,
2 R&D Discovery, Innogenetics NV, Ghent, Belgium,
3 Department of Surgery, Erasmus MC – University Medical Center Rotterdam, Rotterdam, The Netherlands,
4 Department of Biostatistics, Erasmus MC – University Medical Center Rotterdam, Rotterdam, The Netherlands

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ABSTRACT

We studied the influence of a broad range of genetic variants in recipient and donor innate immunity receptors on bacterial and fungal infections and acute rejection after liver transplantation (LT). Seventy-six polymorphisms in TLR 1-10, NOD2, LBP, CD14, MD2, SIGIRR, Ficolins 1, -2, and -3, MASP 1, -2, and -3, and the complement receptor C1qR1 were determined in 188 LT recipients and 135 of their donors. Associations with clinically significant infections and acute rejection were analyzed for 50 polymorphisms. Significant associations were validated in an independent cohort of 181 recipients and 167 donors. Three recipient polymorphisms and 3 donor polymorphisms were associated with infections in the identification cohort, but none of these associations were confirmed in the validation cohort. Three donor polymorphisms were associated with acute rejection in the identification cohort, but not in the validation cohort. In contrast to their effect in the general population, 50 common genetic variations in innate immunity receptors do not influence susceptibility to bacterial/fungal infections after LT. In addition, no reproducible associations with acute rejection after LT were observed. Likely, transplant-related factors play a superior role as risk factors for bacterial/fungal infections and acute rejection after LT.

INTRODUCTION

Lifelong immunosuppressive medication is one of the factors that contribute to the high incidence of infections early after liver transplantation (LT). Currently, infections are the leading cause of death in the first year after LT (1-2). Despite immunosuppressive therapy, about 30% of LT-recipients experience one or more episodes of acute rejection. Apparently, these patients need more immunosuppressive medication, while among 70% of LT-recipients who do not experience acute rejection, a considerable proportion is probably over-immunosuppressed. At present, no tests are available that predict the risk of infection and acute rejection after LT.

Adaptive immunity is inhibited by immunosuppressive therapy. However, innate immunity is largely unaffected by currently used immunosuppressive drugs and is therefore probably important in combating infections after organ transplantation. Innate immune cells recognize molecular structures on pathogens by Pattern Recognition Receptors (PRR), also called innate immunity receptors. Genetic polymorphisms in these receptors have been recently recognized as determinants of the ability to combat infections in non-transplantation patients (3-4). For instance, Toll-like receptor (TLR) 4 rs4986790 and CD14 rs 2569190 polymorphisms have been shown to increase the risk of gram-negative infections in intensive care unit populations (5-8). In a septic shock cohort, the TLR2 rs5743708 polymorphism predisposed patients to staphylococcal infections (9). The same polymorphism may influence the risk of developing tuberculosis (10). In another intensive care unit cohort, polymorphisms in Nucleotide-binding Oligomerization Domain 2 (NOD2) and TLR4 were associated with bacteremia (11).

Little is known about the effects of these and other genetic polymorphisms in innate immunity receptors on the risk of bacterial and fungal infections after organ transplantation. In addition, the available data are often contradictory. An association between a recipient TLR4 single nucleotide polymorphism (SNP) and bacterial infections in kidney transplant recipients (12) was not confirmed in independent studies (13-15). Significant associations between genetic variations in donor Mannose-Binding Lectin 2 (MBL2) and the incidence of infections after LT were reported by two different centers (16-18), but were not confirmed by two other liver transplant centers, including our center (19-20). Apart from the cited studies on the influence of genetic polymorphisms in components of the lectin pathway of complement activation and a recent single-cohort study on one single TLR2 SNP (rs5743708), which showed that this polymorphism did not influence the incidence of gram-positive bacterial infections after LT (21), the relevance of genetic variations in innate immunity receptors for inter-individual differences in susceptibility to bacterial infections after LT has not been studied.

Regarding the influence of polymorphisms in innate immunity receptor genes on acute rejection after LT, less is known. Antigen-presenting cells (APC), which express numerous

PRR, are required for presentation of allo-antigens to T cells, thereby promoting acute rejection, and signalling via PRR in APC dictates the initiation, type, magnitude and duration of T-cell responses (22). Since inflammatory responses upon ischemia-reperfusion are similar to those observed when the innate immune system is activated via PRR and ischemia-reperfusion related injury can contribute to acute rejection, it is likely that PRR play a role in allograft rejection (23). Indeed, PRR can detect sterile injury upon binding to endogenous ligands that are released from damaged tissues (24-26), and thereby activate innate and adaptive immunity. In agreement with this concept, mice that are deficient in TLR signaling display impaired allograft rejection (27-28), and engagement of certain PRR is sufficient to prevent allograft acceptance in experimental animals (29-32). In addition, increased expression of endogenous PRR-ligands has been observed during rejection in allografts in experimental animals (24-25) and in humans (33), and on circulating monocytes (34-35). Moreover, recipient TLR4 SNP have been associated with acute rejection after lung (13, 36) and kidney transplantation (37), although the association in kidney transplantation was not confirmed in two other studies (13, 15). One single study (15) reported a significantly higher rate of acute rejection after kidney transplantation in carriers of the TLR3 rs3775291 SNP T-allele. A CD14 promotor polymorphism was associated with earlier onset of acute rejection in lung transplant recipients (38). However, no studies associating polymorphisms in innate immune receptor genes with acute rejection after LT have been published.

The aim of the present study was to assess whether a broad range of genetic polymorphisms in innate immunity receptors, mostly SNPs, may influence susceptibility to bacterial and fungal infections or development of acute rejection after LT. To prevent the risk of yielding false-positive results, we repeated positive associations in a second cohort, as has been advocated in genetic research (39).

PATIENTS AND METHODS

Study design, patients and setting

The identification cohort consisted of primary LT-recipients who were transplanted at the Erasmus Medical Center, Rotterdam, between 1987 and 2006. We excluded non-Caucasian patients and patients with graft or patient survival less than 5 days, thereby excluding intraoperative and direct postoperative, operation-related morbidity and mortality. From 188 of these patients DNA was available, and from 135 donors of this cohort. Since many innate immunity receptors are expressed in the liver (40) or secreted by the liver, we determined both recipient and donor polymorphisms. While the identification study was performed, we collected DNA from 181 additional primary LT-recipients with a graft and patient survival of at least 5 days, transplanted at the Erasmus Medical

Center between 2001 and 2010 (validation cohort). No patient was included in both cohorts. Polymorphisms that were identified as a risk factor for bacterial and fungal infection and/or rejection after LT in the identification cohort, were subsequently studied in this second cohort, which contained 38 non-Caucasian patients (21 Asians and 17 Black-Africans). From 167 liver donors of this cohort, frozen splenocytes were available, from which donor DNA could be isolated. All patients gave informed consent, and the study was performed according to the guidelines of the Medical Ethics Committee of the Erasmus MC.

Patient follow-up

All patients in the identification and validation cohorts received standard immunosuppressive therapy consisting of corticosteroids and a calcineurin inhibitor (cyclosporine or tacrolimus), with or without mycophenolate mofetil or azathioprine and/or basiliximab. Patients transplanted before 2000 received selective bowel decontamination, using ofloxacin, colistin and amfotericin B. After 2000, patients received cefazolin as antibiotic prophylaxis. After LT, patients were intensively monitored according to the local protocol for any sign of infection and acute rejection. After inclusion in either the identification cohort or the validation cohort, clinical information about bacterial and fungal infections and acute rejection was retrospectively collected from patient files. All infections were categorized into site-specific infections (abdominal infections, bloodstream infections, pneumonia). Clinically serious infections (CSI) were defined using the Center for Disease Control and Prevention criteria (41). Infections were only registered if the etiologic pathogen was identified by culture, and patients received adequate treatment. Infections with only coagulase-negative staphylococci were assumed to be contamination, and excluded. Abdominal infections included cholangitis, peritonitis, and intra-abdominal abscesses, but bilomas were excluded. Pneumonia had to be confirmed by chest X-ray or CT-scan, showing a new or progressive infiltrate. Bacterial infections were grouped by causative pathogen (gram negative bacteria and/or gram positive bacteria). Fungal infections were only included if they were invasive. In addition to bacterial and fungal infection, CMV infection was registrered. CMV infection was defined as positive viral load in blood. Follow-up time for infections was from LT to three months after LT. For acute rejection, the follow-up time was from LT to end of follow-up. End of follow-up was defined as date of acute rejection, date last seen, or date of death. Acute rejection was defined as: at least two-fold increase in serum liver enzymes (AST and/or ALT) or bilirubin, confirmed by a biopsy-proven rejection (rejection activity index (RAI)-score 5 or higher) according to BANFF-criteria (42), and responsiveness to treatment with immunosuppressive medication.

Genotyping

DNA was isolated from peripheral blood of patients or splenocytes from donors using the Wizard® Genomic DNA Purification Kit (Promega), according to the manufacturers' protocol. Seventy-six (76) genetic polymorphisms in 21 signaling and secreted innate immunity receptors were determined in both patient and donor DNA of the identification cohort. Genes studied were TLR 1 to 10, the cytosolic bacterial sensor NOD2, the TLR-accessory molecules LBP, CD14, MD2 and SIGIRR, the Ficolins1, -2, and -3, MASP 1, -2, and -3, and the complement receptor C1qR1. The polymorphism had been selected due to their localization in an exon, an intron (up to 30 bp before an exon; up to 10 bp after an exon) or in a promoter region/5'-untranslated region (UTR) up to 1,000 bp before exon 1. DNA of the samples was amplified using PCR: five multiplex PCR reactions and one single PCR (for NOD2) were carried out. Polymorphisms were determined with a probe assay based on the reverse hybridization principle, using primers and probes developed by Innogenetics NV, Ghent, Belgium. For every SNP, specific probes had been designed both for the major and the minor allele to ensure detection of homozygotes as well as heterozygotes. Hybridization, wash steps, and visualization of the DNA products were performed in Auto-LiPA 30 devices (Innogenetics NV). For each SNP, the dots on the membranes were interpreted using in-house developed software (43).In the validation cohort, SNPs were determined by KBioscience, UK (http://www.kbioscience.co.uk) using the KASP SNP genotyping system, a homogeneous Fluorescent Resonance Energy Transfer (FRET) based system, coupled with competitive allele specific PCR. Twelve polymorphisms in 7 patients and 7 donors of cohort 2 (in total168 samples) were determined by Innogenetics and KBiosciences, which yielded only 1 discrepant result (=0.6%), showing the accuracy of both techniques.

Statistical analysis

Associations between genetic variants and bacterial and fungal infections after LT were determined by chi-square analysis using the outcome "no infection" and "one or more infections" as variables. Associations with "time to first acute rejection episode" were determined by Kaplan-Meier survival analysis, using the Log rank test to assess statistical significance. Associations of baseline characteristics of the LT-patients or their donors with CSI or time to acute rejection were analyzed using Logistic regression and Cox regression, respectively. Analyses were censored for death, re-transplantation, or loss of follow-up. In multivariate analysis, using logistic regression or cox regression respectively, it was tested whether significant associations of polymorphisms with infections or acute rejection were independent of each other and independent of clinical and demographic parameters. Only polymorphisms with at least 10 individuals per genotype were included in multivariate analysis, or genotypes that both contained the risk allele

were combined. Statistical analysis was performed using SPSS for Windows, version 17.0 software package. P-values of <0.05 were considered as statistically significant.

RESULTS

Clinical characteristics and post-transplant infection and rejection

Table 1 shows the clinical and demographic characteristics of both cohorts. The identification cohort consisted of 188 Caucasian primary LT-recipients, of which 68 (36%) had one or more clinically serious infections (CSI) in the first three months after LT, and 60 (32%) experienced one or more episodes of acute rejection. In the validation cohort, 78 (43%) of 181 LT-recipients had one or more bacterial or fungal infections in the first three months after LT, and 47 (26%) experienced acute rejection. In both cohorts most infections were intra-abdominal. The majority of infections was caused by bacteria, in the identification cohort predominantly by gram-positive, but in the validation cohort equally by gram-positive and gram-negative bacteria. None of the clinical and demographic characteristics that were significantly associated with bacterial and fungal infections or acute rejection in the identification cohort could be confirmed in the validation cohort.

Genetic polymorphisms in innate immunity receptors and post-transplant infections

In the identification cohort, genotypes of all 76 polymorphisms were determined in LT-recipients and in their donors (Table 2). Associations with total CSI as well as site-specific CSI (abdominal, bloodstream) were first determined using univariate analysis. Associations with pneumonia as a separate entity were not analyzed, because the number of patients with pneumonia was low. Because many pattern recognition receptors recognize molecular patterns specifically expressed on either gram-negative or gram-positive bacteria, we additionally analyzed associations between genotypes and infections caused by these two different categories of pathogens. No associations with fungal infections as a separate entity were performed because of the low numbers of patients with fungal infections. Subsequently, we included polymorphisms and clinical variables that showed significant association in univariate analysis in a multivariate analysis. To prevent accidental findings, we excluded 26 polymorphisms with less than 10 individuals per genotype from multivariate analysis, unless homozygous and heterozygous carriers of risk alleles emerging from univariate analysis could be combined to a group of at least 10 subjects. Polymorphisms that were included in the analyses are indicated in bold in Table 2. In Table 3 the polymorphisms in recipient and donor innate immunity receptors that were significantly associated with bacterial and fungal infections in the identification cohort in both univariate and multivariate analysis are shown.

Table 1. Clinical variables and infections and rejection in identification and validation cohorts

	Identification cohort								Validation cohort							
	CSI [a]	No CSI	OR	p [b]	Rejection	No rejection	HR	P [c]	CSI	No CSI	OR	p [b]	Rejection	No rejection	HR	P [c]
Variable	(n=68) No. (%)	(n=120) No. (%)			(n=60) No. (%)	(n=128) No. (%)			(n=78) No. (%)	(n=103) No. (%)			(n=47) No. (%)	(n=134) No. (%)		
Age recipient (median, range)	45 (16-66)	47 (16-68)	1.01	0.396	45 (19-67)	46 (16-68)	0.99	0.368	48 (17-67)	50 (16-69)	0.99	0.540	45 (18-69)	51 (16-66)	0.97	**0.033**
Age donor (median, range)	38 (12-62)	39 (11-68)	0.99	0.637	39 (12-64)	38 (11-68)	1.02	0.062	45 (13-78)	47 (14-77)	0.99	0.540	46 (16-66)	46 (13-78)	1.00	0.949
Gender recipient																
Male	36 (36)	64 (64)	1.00	0.650	27 (27)	73 (73)	1.00	0.813	51 (43)	67 (57)	1.00	0.813	29 (25)	89 (75)	1.00	0.731
Female	32 (36)	56 (64)	0.86		33 (38)	55 (62)	0.93		27 (43)	36 (57)	0.92		18 (29)	45 (71)	1.12	
Gender donor																
Male	25 (28)	65 (72)	1.00	**0.034**	32 (36)	58 (64)	1.00	0.226	35 (40)	52 (60)	1.00	0.551	20 (23)	67 (77)	1.00	0.384
Female	43 (44)	55 (56)	1.98		28 (29)	70 (71)	0.72		43 (46)	51 (54)	1.22		27 (29)	67 (71)	1.31	
Ethnicity																
Caucasian	68 (36)	120 (64)			60 (32)	128 (68)			57 (40)	86 (60)	1.00	0.523	37 (26)	106 (74)	1.00	0.307
Black									9 (53)	8 (47)	1.50		5 (29)	12 (71)	0.86	
Asian									12 (57)	9 (43)	1.67		5 (24)	16 (76)	0.44	
Underlying disease[d]																
Viral hepatitis	11 (28)	28 (72)	1.00	0.386	7 (18)	32 (82)	1.00	**0.023**	19 (50)	19 (50)	1.00	0.984	10 (26)	28 (74)	1.00	0.074
Cholestatic/ Autoimmune	25 (40)	38 (60)	1.74		24 (38)	39 (62)	2.51		23 (42)	32 (58)	0.84		18 (33)	37 (67)	0.94	
Alcoholic disease	5 (25)	15 (75)	0.85		7 (35)	13 (65)	2.08		12 (41)	17 (59)	1.01		4 (14)	25 (86)	0.50	
Acute fulminant	8 (33)	16 (67	1.75		13 (54)	11 (46)	4.22		1 (50)	1 (50)	1.06		2 (100)	0 (0)	12.3	
Other	19 (45)	23 (55	2.23		9 (21)	33 (79)	1.22		23 (40)	34 (60)	0.79		13 (23)	44 (77)	0.70	
Year of LT																

Table 1. Clinical variables and infections and rejection in identification and validation cohorts (continued)

	Identification cohort								Validation cohort							
	CSI [a]	No CSI	OR	p [b]	Rejection	No rejection	HR	P [c]	CSI	No CSI	OR	p [b]	Rejection	No rejection	HR	P [c]
Variable	(n=68) No. (%)	(n=120) No. (%)			(n=60) No. (%)	(n=128) No. (%)			(n=78) No. (%)	(n=103) No. (%)			(n=47) No. (%)	(n=134) No. (%)		
Before 1995	14 (36)	25 (64)	1.00	0.959	18 (46)	21 (53)	1.00	**0.006**	29 (57)	22 (43)	1.00	0.119	13 (26)	38 (74)	1.00	0.816
1995-1999	27 (37)	47 (63)	0.89		25 (34)	49 (66)	0.70		13 (35)	24 (65)	0.43		10 (27)	27 (73)	0.85	
After 2000	27 (36)	48 (64)	0.90		17 (23)	58 (77)	0.33		36 (39)	57 (61)	0.53		24 (26)	69 (74)	1.10	
Acute Rejection																
No	45 (35)	83 (65)	1.00	0.712					60 (45)	74 (55)	1.00	0.432				
Yes	23 (38)	37 (62)	1.14						18 (38)	29 (62)	0.75					
CMV infection																
No	55 (34)	109 (66)	1.00	0.095	49 (30)	115 (70)	1.00	0.051								
Yes	13 (54)	11 (46)	2.21		11 (46)	13 (54)	2.09									

a Numbers of different types of infections: Identification cohort: abdominal 48, Pneumonia 15, Bloodstream 31, Gram positive 57, Gram negative 38, Fungal 13. Validation cohort: abdominal: 51, Pneumonia 17, Bloodstream 44, Gram positive 49, Gram negative 52, Fungal 25. (Total of numbers of different types of infections is not equal to total CSI because one individual could have more than one type of infection)b P values for Logistic regression test (multivariate analysis), using yes/no infection as outcome c P values for Cox regression test (multivariate analysis), using time to first rejection as outcomed Viral hepatitis: chronic hepatitis B or C infection; Cholestatic/Autoimmune: Primary Sclerosing Cholangitis, Primary Biliary Cirrhosis, Autoimmune hepatitis; Acute fulminant: Acute liver failure
CSI: Clinically Serious Infection (all types); CMV: Cytomegalovirus OR: Odds ratio; HR: Hazard ratio

Table 2. Genetic polymorphisms in innate immune receptors determined in the identification cohort

			Identification cohort	
Gene	**rs number**	**Region**	**Genotypes donors (no.)**	**Genotypes recipients (no.)**
Masp1-3	**rs710474**	**Promoter**	**CC/CT/TT 57/60/15**	**CC/CT/TT 87/85/16**
Masp3	**rs850312**	**Protease domein**	**GG/GA/AA 56/60/16**	**GG/GA/AA 90/76/22**
Masp1	**rs3733001**	**Intron 15**	**GG/GA/AA 83/41/9**	**GG/GA/AA 112/66/10**
Masp2	not submitted	Exon3 D105	AA/AG/GG 120/12/0	AA/AG/GG150/29/9
Masp2	rs2273343	Exon4	AA/AG 132/0	AA/AG 187/1
Masp2[a]	**rs6695096**	**Intron 8**	**AA/AG/GG 92/38/2**	**AA/AG/GG 140/42/6**
Masp2	rs223346	Exon 9	TT/CT 128/3	TT/CT 183/4/1
Masp2[a]	**rs12711521**	**Exon 9**	**TT/TG/GG 88/38/6**	**TT/TG/GG 134/46/8**
Masp2[a]	**rs1782455**	**Exon 11**	**TT/CT/CC 92/34/6**	**TT/CT/CC 138/44/6**
FCN1	**rs2989727**	**Promoter**	**AA/GA/GG 52/62/18**	**AA/GA/GG 85/78/25**
FCN1	**rs1071583**	**Exon 9**	**GG/AG/AA 56/59/17**	**GG/AG/AA 89/75/24**
FCN2	rs7865453	Promoter	AA/AC/CC 101/30/1	AA/AC/CC 148/37/3
FCN2	**rs17514136**	**Promoter**	**AA/AG/GG 64/57/11**	**AA/AG/GG 85/90/13**
FCN2	**rs17549193**	**Exon 8**	**CC/CT/TT 62/55/15**	**CC/CT/TT 76/96/16**
FCN2	rs7851696	Exon 8	GG/GT/TT 93/38/2	GG/GT/TT 143/42/3
FCN3	rs3813800	Intron 5	CC/CG 132/0	CC/CG 187/1
C1qR1	**rs3746731**	**Exon 1**	**TT/CT/CC 46/62/24**	**TT/CT/CC 66/89/33**
C1qR1	rs7492	Exon 2 (untranslated region)	CC/CT/TT 116/16/1	CC/CT/TT 157/30/1
LBP	rs2232578	Promoter	AA/AG/GG 92/36/4	AA/AG/GG 115/65/8
LBP	rs5744204	Exon 4	GG/AG 128/4	GG/AG 185/3
LBP	rs2232607	Exon 8	AA/AG 130/2	AA/AG 186/2
LBP	**rs1780627**	**Intron 9**	**CC/TC/TT 28/77/27**	**CC/TC/TT 41/116/31**
LBP[a]	**rs2232613**	**Exon 10**	**CC/CT/TT 109/19/4**	**CC/CT/TT 163/25/0**
LBP	**rs2232618**	**Exon 13**	**TT/TC 117/15**	**TT/TC 154/34**
CD14[a]	**rs3138078**	**Promoter**	**GG/GT/TT 77/51/4**	**GG/GT/TT 112/70/6**
CD14	**rs2569190**	**Promoter**	**CC/TC/TT 39/63/28**	**CC/TC/TT 38/102/48**
CD14	**rs2228049**	**Exon 2**	**AA 132**	**AA 188**
CD14	**rs2563298**	**Intron 9**	**GG/GT/TT 62/58/12**	**GG/GT/TT 109/68/11**
MD2	**rs1809440**	**Promoter**	**AA/AG/GG 48/54/30**	**AA/AG/GG 65/84/39**
MD2	**rs6472812**	**Exon 2**	**GG/AG 117/15**	**GG/AG 171/17**
MD2[b]	**rs11466004**	**Exon 5**	**CC/CT 130/2**	**CC/CT 175/13**
NOD2	**rs2066844**	**Exon 4**	**CC/CT 123/9**	**CC/CT 166/22**
NOD2	rs2066845	Exon 8	GG/GC 130/2	GG/GC 185/3
NOD2	rs2066847	Exon 11 (insertion)	—/-C 127/5	—/-C 180/8
Sigirr	rs7482596	Promoter	AA 131	AA 188
Sigirr[a]	**rs3210908**	**Exon 9**	**GG/AG/AA 75/48/7**	**GG/AG/AA 106/64/18**

Table 2. Genetic polymorphisms in innate immune receptors determined in the identification cohort (continued)

			Identification cohort	
Gene	**rs number**	**Region**	**Genotypes donors (no.)**	**Genotypes recipients (no.)**
Sigirr[a]	**rs3087588**	**Exon 9**	**GG/TG/TT 75/48/7**	**GG/TG/TT 106/64/18**
TLR1	**rs5743551**	**Promoter**	**AA/AG/GG 68/57/8**	**AA/AG/GG 100/66/22**
TLR1	rs5743611	Exon4	GG/GC/CC 111/19/3	GG/GC/CC 163/24/1
TLR1	**rs4833095**	**Exon4**	**AA/AG/GG 69/57/8**	**AA/AG/GG 101/65/22**
TLR1	rs3923647	Exon4	AA/AT/TT 123/10/1	AA/AT/TT 182/6/0
TLR2	**rs1898830**	**Promoter**	**AA/AG/GG 52/59/21**	**AA/AG/GG 77/89/22**
TLR2	rs3804100	Exon 2	TT/TC/CC 111/23/0	TT/TC/CC 165/21/2
TLR2	**rs5743708**	**Exon2**	**GG/GA 129/5**	**GG/GA 176/12**
TLR2	not submitted	Exon2 R677W	CC 134	CC 188
TLR3	**rs5743305**	**Promoter**	**TT/TA/AA 59/57/18**	**TT/TA/AA 69/91/28**
TLR3	rs3775296	Intron 1	CC/CA/AA 88/40/6	CC/CA/AA 133/48/7
TLR3	**rs3775291**	**Exon4**	**CC/CT/TT 62/60/12**	**CC/CT/TT 103/71/14**
TLR4	**rs1927914**	**Promoter**	**AA/GA/GG 69/50/15**	**AA/GA/GG 83/81/24**
TLR4	**rs4986790**	**Exon4**	**AA/AG 117/17**	**AA/AG 165/23**
TLR4	**rs4986791**	**Exon4**	**CC/CT 116/18**	**CC/CT 165/23**
TLR5	**rs759303**	**Promoter**	**GG/GA 121/13**	**GG/GA 170/18**
TLR5	rs5744168	Exon6	CC/CT/TT 118/12/3	CC/CT/TT 170/17/1
TLR5	rs2072493	Exon6	AA/AG/GG 105/24/4	AA/AG/GG 139/45/4
TLR5	**rs5744174**	**Exon6**	**TT/CT/CC 45/55/33**	**TT/CT/CC 56/100/32**
TLR6	**rs1039559**	**Promoter**	**TT/CT/CC 36/69/29**	**TT/CT/CC 59/84/45**
TLR6	**rs5743810**	**Exon1**	**CC/CT/TT 43/67/24**	**CC/CT/TT 77/74/37**
TLR6	**rs3821985**	**Exon1**	**CC/CG/GG 55/65/13**	**CC/CG/GG 88/82/18**
TLR6	rs5743815	Exon1	TT/CT 125/8	TT/CT 183/5
TLR7	rs5743712	Promoter	GG/GC 134/0	GG/GC 186/2
TLR7	**rs2897827**	**Promoter**	**AA/AT/TT 93/27/14**	**AA/AT/TT 128/30/30**
TLR7	rs179008	Exon3	CC/CT 131/2	CC/CT 187/1
TLR7	rs5743781	Exon3	CC/CT 130/2	CC/CT 187/1
TLR8	**rs1548731**	**pro(var1)/IVS1 (var2)**	**CC/CT/TT 98/20/15**	**CC/CT/TT 119/32/37**
TLR8	**rs3764880**	**UTR5 (var1)/Exon1(var2)**	**AA/AG/GG 94/24/16**	**AA/AG/GG 125/34/29**
TLR8	rs5744077	Exon3	AA/AG/GG 132/0/0	AA/AG/GG 180/4/4
TLR8	**rs2159377**	**Exon3**	**CC/CT/TT 100/23/10**	**CC/CT/TT 138/31/19**
TLR8	**rs5744080**	**Exon3**	**CC/CT/TT 78/28/32**	**CC/CT/TT 94/39/55**
TLR9	**rs187084**	**Promoter**	**TT/CT/CC 41/67/25**	**TT/CT/CC 64/96/28**
TLR9	rs5743836	Promoter	TT/TC/CC 98/30/5	TT/TC/CC 125/55/8
TLR9	**rs352140**	**Exon2**	**AA/AG/GG 44/61/29**	**AA/AG/GG 58/91/39**
TLR10	**rs7694115**	**Promoter**	**TT/CT/CC 57/61/16**	**TT/CT/CC 73/78/37**

Table 2. Genetic polymorphisms in innate immune receptors determined in the identification cohort (continued)

			Identification cohort	
Gene	**rs number**	**Region**	**Genotypes donors (no.)**	**Genotypes recipients (no.)**
TLR10[a],[b]	**rs11466645**	**Promoter**	**TT/TA/AA 86/45/1**	**TT/TA/AA 116/50/22**
TLR10[a]	**rs11096957**	**Exon3**	**AA/AC/CC 56/60/18**	**AA/AC/CC 75/77/36**
TLR10[a],[b]	**rs11096956**	**Exon3**	**GG/GT/TT 77/54/3**	**GG/GT/TT 107/59/22**
TLR10	**rs4129009**	**Exon3**	**AA/AG/GG 87/44/2**	**AA/AG/GG 118/57/13**

[a] Genotypes containing minor alleles were combined, based on outcomes of univariate analysis
[b] Numbers of genotypes significantly different between donors and recipients ($p<0.05$)
Bold polymorphisms were included in analyses, based on number of subjects per genotype of at least 10 or when minor alleles could be combined to a group of at least 10 if significantly associated in univariate analysis.
Abbreviations of the genes: MASP: MBL-associated serine protease; FCN: ficolin; C1qR1: Complement 1 q receptor 1; LBP: lipopolysaccharide-binding protein; CD14/MD2: co-receptors; NOD: Nucleotide-binding oligomerization domain; TLR: Toll-like receptor

Recipient polymorphisms

The T-allele of recipient TLR10 gene polymorphism rs7694115 was protective against abdominal infections. LT-recipients that carried the Lipopolysaccharide Binding Protein (LBP) gene rs2232613 T-allele were completely protected from bloodstream infections. Recipients carrying the C-allele of the TLR3 rs3775291 polymorphism were less susceptible to infections caused by gram-negative micro-organisms.

Donor polymorphisms

Two donor polymorphisms were significantly associated with the incidence of total CSI: CD14 rs2569190 and TLR9 rs187084. The T-allele of the CD14 SNP was protective against total CSI, and was also significantly associated with a lower incidence of infections caused by gram-positive bacteria. Patients transplanted with livers from donors homozygous for the TLR9 rs187084 T-allele were protected from total CSI, bloodstream infections, and gram-negative infections. Patients who received a liver from a donor with an A-allele in the Sigirr rs3210908 gene had a higher incidence of bloodstream infections and gram-negative infections compared to the GG-genotype.

The results of genotyping of those significantly associated polymorphisms in the validation cohort are shown in Table 4, while associations with infections are shown in Table 3. None of the associations observed in the identification cohort was confirmed in the second cohort. While the identification cohort consisted of Caucasian patients only, the validation cohort contained 38 non-Caucasian patients. Therefore, we repeated the analyses in Caucasians only, but again none of the polymorphisms showed significant association with infections.

Table 3. Genetic polymorphisms significantly associated with infections after LT in the identification cohort and their associations in the validation cohort

			Identification cohort				Validation cohort[d]		
Gene and SNP	Type of infection	Genotype	Infection no. (%)	No infection no. (%)	Univariate analysis [a] (p-value)	Multivariate analysis [b] (p-value)	Infection no. (%)	No infection no. (%)	Univariate analysis [a] (p-value)
Recipient SNP									
TLR10 rs7694115	ABD	CC	15 (41)	22 (59)	0.019	0.012	3 (19)	13 (81)	0.560[c]
		TT+CT	33 (22)	118 (78)			37 (28)	96 (72)	
LBP rs2232613	BLOOD	CC	31 (19)	132 (81)	0.017	0.002	35 (25)	107 (75)	0.970
		CT	0 (0)	25 (100)			6 (25)	18 (75)	
TLR3 rs3775291	GRAM$^-$	TT	7 (50)	7 (50)	0.009	0.007	2 (29)	5 (71)	1.000[c]
		CC+CT	31 (18)	143 (82)			41 (28)	104 (72)	
Donor SNP									
CD14 rs2569190	CSI	CC	19 (49)	20 (51)	0.043	0.033	16 (39)	25 (61)	0.519
		TC+TT	27 (30)	64 (70)			52 (45)	64 (55)	
TLR9 rs187084	CSI	TT	8 (20)	33 (80)	0.024	0.039	17 (35)	32 (65)	0.318
		CT	30 (45)	37 (55)			39 (46)	46 (54)	
		CC	9 (36)	16 (64)			16 (50)	16 (50)	
Sigirr rs3210908	BLOOD	GG	8 (11)	67 (89)	0.010	0.032	22 (24)	71 (76)	0.995
		AG+AA	15 (27)	40 (73)			17 (24)	55 (76)	
Sigirr rs3210908	GRAM$^-$	GG	9 (12)	66 (88)	0.002	0.003	24 (26)	69 (74)	0.291
		AG+AA	17 (31)	38 (69)			24 (33)	48 (67)	
TLR9 rs187084	BLOOD	TT	0 (0)	41 (100)	0.000	0.000	12 (25)	37 (75)	0.807
		CT	18 (27)	49 (73)			19 (22)	66 (78)	
		CC	6 (24)	19 (76)			9 (28)	23 (72)	

Table 3. Genetic polymorphisms significantly associated with infections after LT in the identification cohort and their associations in the validation cohort (continued)

			Identification cohort				Validation cohort[d]		
Gene and SNP	Type of infection	Genotype	Infection no. (%)	No infection no. (%)	Univariate analysis [a] (p-value)	Multivariate analysis [b] (p-value)	Infection no. (%)	No infection no. (%)	Univariate analysis [a] (p-value)
TLR9 rs187084	GRAM$^-$	TT	2 (5)	39 (95)	0.003	0.027	12 (25)	37 (75)	0.503
		CT	20 (30)	47 (70)			28 (33)	57 (67)	
		CC	5 (20)	20 (80)			8 (25)	24 (75)	
CD14 rs2569190	GRAM$^+$	CC	18 (46)	21 (54)	0.011	0.011	7 (17)	34 (83)	0.103
		TC+TT	20 (22)	71 (78)			35 (30)	81 (70)	

[a] P values for Chi^2 test, using yes/no infection as outcome

[b] P values for Logistic regression test, using yes/no infection as outcome. SNP were tested together with significantly associated clinical variables.

[c] P values for Fisher exact test

ABD: abdominal infection; BLOOD: bloodstream infection; PNEU: Pneumonia; GRAM$^-$: infection with gram negative micro-organism; GRAM$^+$: infection with gram positive micro-organism; CSI: Clinically Serious Infection (all types)

[d] Due to limited amounts of DNA available, some polymorphisms could not be determined in all recipients and donors included in the validation cohort.

Table 4. Genetic polymorphisms in innate immune receptors determined in the validation cohort

Gene	rs number	Region / for MBL level: donor genotypes	Validation cohort	
			Genotypes donors (no.)	Genotypes recipients (no.)
LBP	rs2232613	Exon 10	CC/CT/TT 137/29/1	CC/CT/TT 142/24/0
Sigirr	rs3210908	Exon 9	GG/AG/AA 93/62/10	GG/AG/AA 113/46/5
CD14	Rs2569190	Promoter	CC/CT/TT 41/81/35	CC/CT/TT 37/87/37
TLR1	rs5743551	Promoter	AA/AG/GG 84/59/14	AA/AG/GG 75/69/17
TLR3	rs3775291	Exon 4		CC/CT/TT 84/61/7
TLR6	rs5743810	Exon1	CC/CT/TT 57/77/29	CC/CT/TT 76/62/23
TLR9	rs187084	Promoter	TT/CT/CC 49/85/32	
TLR9	rs352140	Exon2	TT/CT/CC 41/54/29	
TLR10	rs7694115	Promoter		AA/GA/GG 58/75/16

Genetic polymorphisms in innate immunity receptors and acute rejection

Analyses of associations with acute rejection were performed in a similar manner as described for assocations with infections. In Table 5 the polymorphisms that were significantly associated in both univariate analysis and multivariate analysis with acute rejection in the identification cohort are shown.

Recipient polymorphisms

No recipient polymorphisms were associated with acute rejection after LT.

Donor polymorphisms

Three donor polymorphisms were significantly associated with acute rejection in the identification cohort. The minor T-allele of the donor LBP rs2232613 gene was associated with a higher incidence of acute rejection. In the donor TLR6 rs5743810 gene the T-allele was a risk allele for acute rejection with a dose-allele effect on the incidence of acute rejection. The donor TLR9 rs352140 G-allele was associated with a higher incidence of acute rejection.

The results of the replication of these associations in the validation cohort are also depicted in Table 5, showing that none of the associations was confirmed. Likewise, no significant association was obtained when the analyses were repeated for Caucasian patients only.

Table 5. Genetic polymorphisms significantly associated with acute rejection after LT in the identification and their associations in the validation cohort

		Identification cohort				Validation cohort[c]		
Gene and SNP	**Genotype**	**Rejection no. (%)**	**No rejection no. (%)**	**Univariate analysis [a] (p-value)**	**Multivariate analysis [b] (p-value)**	**Rejection no. (%)**	**No rejection no. (%)**	**Univariate analysis [a] (p-value)**
Recipient SNP								
No SNP associated								
Donor SNP								
LBP rs2232613	CC	24 (22)	85 (78)	0.000	0.031	37 (27)	100 (73)	0.977
	CT+TT	13 (57)	10 (43)			8 (27)	22 (73)	
TLR6 rs5743810	CC	5 (12)	38 (88)	0.004	0.001	13 (23)	44 (77)	0.775
	CT	22 (33)	45 (67)			22 (29)	55 (71)	
	TT	11 (46)	13 (54)			8 (28)	21 (72)	
TLR9 rs352140	AA	6 (14)	38 (86)	0.025	0.008	8 (20)	33 (80)	0.658
	AG	23 (38)	38 (62)			15 (28)	39 (72)	
	GG	9 (31)	20 (69)			8 (28)	21 (72)	

[a] P values for Log rank test, using time to first rejection as outcome

[b] P values for Cox regression test with backward selection procedure, using time to first rejection as outcome. SNP were tested together with significantly associated clinical variables

[c] Due to limited amounts of DNA available, some polymorphisms could not be determined in all recipients and donors included in the validation cohort.

DISCUSSION

To determine whether genetic variations in innate immunity receptors may influence susceptibility to bacterial and fungal infections and rejection after LT, we designed a comprehensive study to identify possible associations between genetic polymorphisms in all known TLRs, TLR4 accessory molecules (LBP, CD14, MD2, SIGIRR), the NOD-like receptor NOD2, and components of the lectin pathway of complement activation (Ficolins, MBL-associated serine proteases (MASPs), and C1q receptor 1) in both recipients and donors with post-transplant infections and acute rejection. To prevent the pitfall of false-positive associations due to a type I error, we repeated the observed associations in a second cohort from the same liver transplant center. We did not include MBL polymorphisms in the present study, since we have previously observed that these are not associated with infections in LT-patients from our center (20). Several polymorphisms were significantly associated with the risk of bacterial or fungal infection or acute rejection after LT in our identification cohort, but none of these associations was confirmed in the validation cohort. Even the association between donor TLR9 rs 187084 with bloodstream infections, which after adjustment for multiple testing according to Bonferroni had a two-sided p-value <0.05 in the identification cohort, was not confirmed in the second cohort. Interestingly, donor MASP3 rs12711521 and Ficolin-2 rs17549193 SNP, which were recently found to be associated with post-LT bacterial infections in another center (18) showed in the present study no association with bacterial infections. In addition, none of the donor polymorphisms that were significantly associated with acute rejection in the identification cohort could be confirmed in the validation cohort. Our study demonstrates the absolute necessity of validating genetic associations in an independent cohort before drawing conclusions.

Interestingly, several of the polymorphisms in innate immunity receptor genes analyzed in the present study have been associated with alterations in susceptibility to bacterial or fungal infections in critically ill non-transplant patients (3-11). None of these polymorphisms were associated with infections after LT in the present study. Apparently, the influence of genetic variations in innate immunity receptors of recipients or donors on the occurrence of post-transplant bacterial and fungal infections is less important compared to transplant-related factors. The impact of surgical trauma combined with the often dismal clinical condition of recipients and the use of immunosuppressive drugs render patients at high risk for infections early after LT. Furthermore, not much is known about the influence of type and intensity of immunosuppression on expression of PRR, although it has been shown that high dose steroid pulse therapy to treat acute rejection after LT reduced expression of TLR2 and TLR4 on circulating monocytes (35). Such effects of immunosuppressive drugs may influence the ability of combat infections. The incidence of CSI after LT is severely influenced by high volumes of blood transfusion and

by prolonged intensive care stay (2, 44-46). Cytomegalovirus (CMV) seropositive donor, prolonged operative procedure time, bilio-enteric reconstruction, and biliary stricturing after LT are risk factors for bacteremia (47-48). Pneumonia is more frequent after LT than after other transplantations (49). In addition to the usual operative risks, recipients of LT are at higher risk for pneumonia due to pre-existing alterations in the diaphragm function, pleural effusion and atelectasis related to ascites, blood product transfusions, immunosuppression and pulmonary involvement associated with their pre-LT underlying liver disease (50). Other risk factors for pneumonia after LT described in the literature are: piggyback anastomosis, mechanical ventilation, lactatemia, vasopressor requirements and lung edema (50-52). These transplant-related factors strongly increase the risk of post-LT infections, and may have more impact than differences in genetic susceptibility to infections after LT.

The present study has a few limitations. At first, the numbers of patients in our identification cohort may have been too low to detect associations with alleles with low allele frequencies. For this reason, we excluded polymorphisms with less than 10 individuals per genotype in the identification cohort from multivariate analysis, unless homozygous and heterozygous carriers of risk alleles emerging from univariate analysis could be combined to a group of at least 10 subjects. As a result, the conclusions of our study apply only for 50 more common polymorphisms that are indicated in bold in Table 2. Nevertheless, we detected in the identification cohort statistically significant associations of 3 recipient SNPs and 3 donor SNPs with infections, and of 3 donor SNPs with acute rejection, of which one (LBP rs2232613) had a minor allele frequency of only 0.10. Secondly, the use of unselected LT-patients with different underlying liver diseases may have introduced confounding factors that disturb penetration of the effects of genetic variations on outcome. However, this may also be considered as strength of the study, because results are representative for a general LT-population. Thirdly, LT-patients from which no DNA was available were excluded from the cohorts. However, since 73 of 76 SNPs showed comparable genotype distributions in recipients and donors (Table 2), we believe that this limitation has not resulted in a genetic bias of the cohorts.

In conclusion, our data together with a previously published study (20) show that neither risk of bacterial and fungal infections nor risk of acute rejection after LT is significantly influenced by 50 common genetic polymorphisms in innate immunity receptors. Transplant-related variables may play a superior role as risk factors for bacterial and fungal infections and acute rejection compared to the genetic polymorphisms tested.

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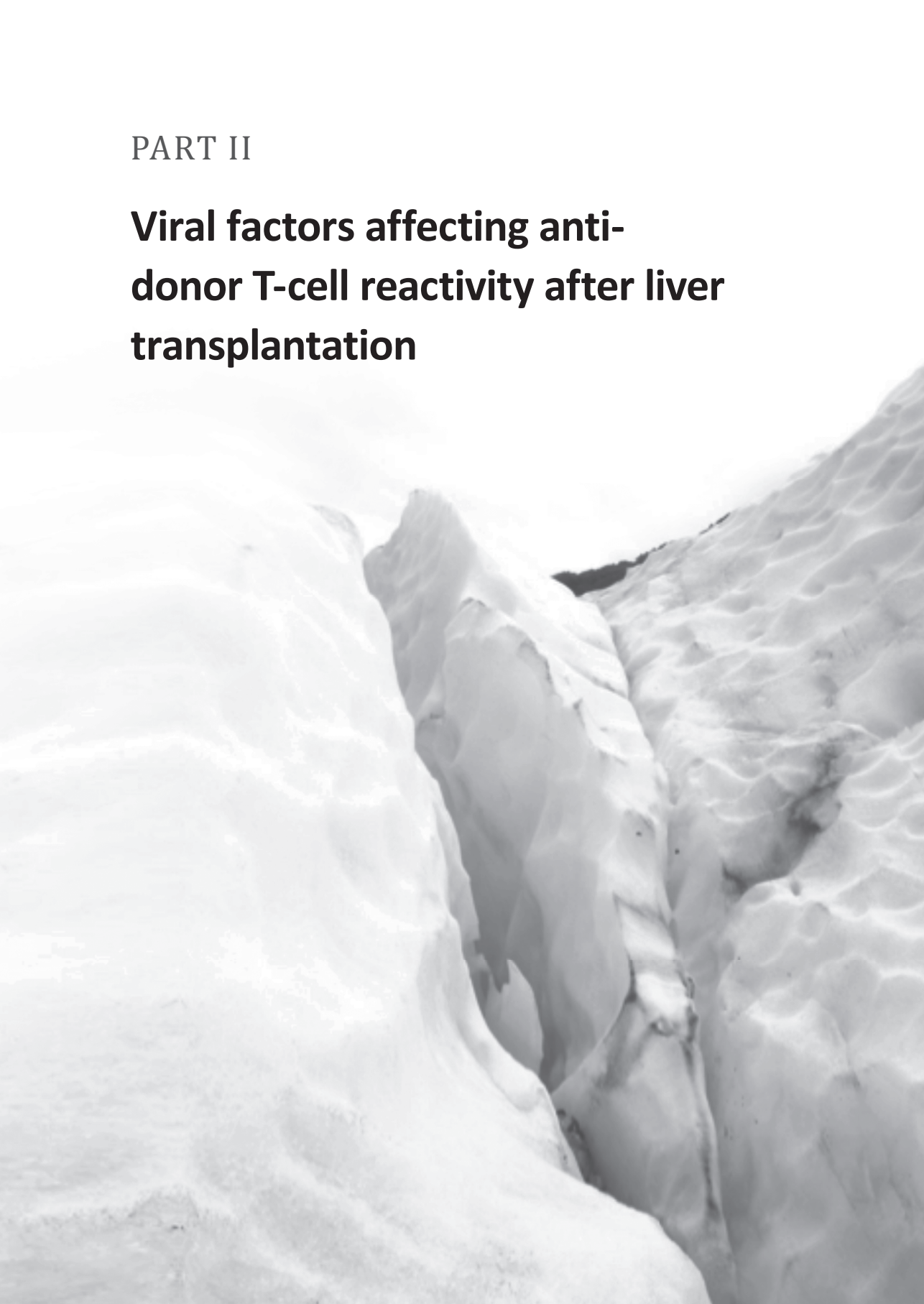
PART II

Viral factors affecting anti-donor T-cell reactivity after liver transplantation



CHAPTER 5

CMV primary infection is associated with donor-specific T-cell hyporesponsiveness and fewer late acute rejections after liver transplantation

X-L. Shi[1,5]
E.L.D. de Mare-Bredemeijer[1]
Ö. Tapirdamaz[1]
B.E. Hansen[1]
R. van Gent[1]
M.J.H. van Campenhout[1]
S. Mancham[1]
N.H.R. Litjens[2]
M.G.H. Betjes[2]
A.A. van der Eijk[3]
Q. Xia[5]
L.J.W. van der Laan[4]
J. de Jonge[4]
H.J. Metselaar[1]
J. Kwekkeboom[1]

1 Department of Gastroenterology and Hepatology, Erasmus MC - University Medical Center, Rotterdam, The Netherlands
2 Department of Internal Medicine, Erasmus MC - University Medical Center, Rotterdam, The Netherlands
3 Department of Virology, Erasmus MC - University Medical Center, Rotterdam, The Netherlands
4 Department of Surgery, Erasmus MC - University Medical Center, Rotterdam, The Netherlands
5 Department of Liver Surgery, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

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ABSTRACT

Viral infections, including cytomegalovirus (CMV), abrogate transplantation tolerance in animal models. Whether this also occurs in humans remains elusive. We investigated how CMV affects T cells and rejection episodes after liver transplantation (LT). Phenotype and alloreactivity of peripheral and allograft-infiltrating T cells from LT patients with different CMV status were analyzed by flow cytometry. The association of CMV status with early and late acute rejection was retrospectively analyzed in a cohort of 639 LT patients. CMV-positivity was associated with expansion of peripheral effector memory T-cell subsets after LT. Patients with CMV primary infection showed donor-specific $CD8^+$ T-cell hyporesponsiveness. While terminally differentiated effector memory cells comprised the majority of peripheral donor-specific $CD8^+$ T cells in CMV primary infection patients, they were rarely present in liver allografts. Retrospective analysis showed that R-D+ serostatus was an independent protective factor for late acute rejection by multivariate Cox regression analysis (hazard ratio=0.18, 95%CI=0.04-0.86, P=0.015). Additionally, CMV primary infection patients showed the highest Vδ1/Vδ2 γδ T-cell ratio, which has been shown to be associated with operational tolerance after LT. In conclusion, our data suggest that CMV primary infection may promote tolerance to liver allografts, and CMV status should be considered when tapering or withdrawing immunosuppression.

INTRODUCTION

Cytomegalovirus (CMV) is a prevalent β-herpesvirus that establishes lifelong latency in humans, and a leading viral infection after solid organ transplantation (1). Immune responses to viruses, including CMV, have been proposed as one of the main barriers to the achievement of transplantation tolerance (2) as they prevent tolerance induction in experimental animal models (3-5). However, associations between CMV infection and graft rejection in humans vary between different types of organ transplants and show inconsistent results (6). No consensus has been made on the effect of CMV infection on graft rejection or tolerance after liver transplantation (LT).

Acute rejection (AR) is primarily initiated by recipient T lymphocytes (T cells) that recognize nonself antigens derived from donor (7). T cells can be classified into different subsets based on their differentiation status, reflecting distinct migration patterns and effector functions upon antigenic stimulation. While naive (TNaive) and central memory (TCM) T cells proliferate robustly in response to antigen, effector memory (TEM) and terminally differentiated effector memory (TEMRA) T cells produce high amounts of pro-inflammatory cytokines and cytolytic mediators (8, 9). In addition, TNaive and TCM recirculate between secondary lymphoid organs, while TEM home to inflamed peripheral tissues (10). On one hand, accumulation of $CD8^+$ TEMRA is a typical characteristic of CMV-driven immune senescence (11), which is associated with increased susceptibility of the elderly to infections, and poor responses to vaccinations (12, 13). On the other hand, CMV-specific memory T cells are hypothesized to be detrimental to allografts as they can be cross-reactive to allogeneic HLA (14, 15). However, no literature is available on how CMV infection alters T-cell alloreactivity after LT.

To address these issues, using *ex vivo* isolated cells from peripheral blood and liver allografts explanted during re-LT, we investigated the effect of CMV infection on T-cell differentiation and alloreactivity, as well as on γδ T-cell subset distribution, which has been shown to be associated with operational tolerance after LT (16-18). In addition, we performed a retrospective study in a cohort of 639 LT patients, to analyze the association of CMV infection with both early (< 6 months) and late (> 6 months) acute rejection (EAR and LAR).

PATIENTS AND METHODS

Study subjects

Peripheral blood samples were collected from 75 patients that underwent primary orthotopic LT between 2009 and 2012 at Erasmus MC, The Netherlands (Supplemental

Table 1). Liver allograft biopsies were obtained from explants of 10 patients that underwent re-LT (Supplemental Table 2), and 9 heathy donor livers prior to LT.

In the retrospective analysis to study the impact of CMV on graft rejection, 639 patients that underwent LT at Erasmus MC from 1992-2010 were included. Demographic details of donors and recipients are summarized in Table 1. Patients were followed up until graft loss, death, or the end of the study period on 31 December 2011.

Immunosuppression therapies are described in Supplemental Materials and Methods.

Written informed consent was obtained from all patients before collection of samples. The medical ethics committee of the Erasmus MC approved this study.

Cell isolation and flow cytometry

Protocols for cell isolation, list of antibodies, and flow cytometry details are described in Supplemental Materials and Methods.

Quantification of alloreactive T-cell frequencies

Alloreactive T cells were analyzed by determination of activation-induced CD137 expression after allogeneic stimulation (19, 20)(Supplemental Figure 1). Protocol is described in Supplemental Materials and Methods.

CMV diagnostics, treatment, and patient groups

CMV serostatus of patients and donors was determined as part of the standard diagnostic routine. CMV viremia was determined by CMV-DNA polymerase chain reaction (PCR) assay, and 50 copies/mL was considered as the threshold for positive result. Patients included in the *ex vivo* T-cell analysis were grouped as: CMV-negative (R-D-), primary infection (R-D^+), and R^+ patients. CMV primary infection was confirmed by detection of viremia or IgG seroconversion after LT, and CMV-negative was defined as no detection of viremia nor IgG seroconversion prior to blood collection (Supplemental Table 1). No differences in baseline characteristics were observed between groups in all experiments. Detailed CMV diagnostics and treatment strategies are available in Supplemental Materials and Methods.

Definition of early and late acute rejection

AR was defined as graft dysfunction accompanied by moderate or severe rejection activity (RAI≥5) detected in the liver biopsy according to Banff criteria, and responsiveness to additional immunosuppressive treatment. While EAR was defined as rejection occurring within 180 days after LT, LAR was defined as those occurring after 180 days after LT. Associations of CMV with EAR or LAR were analyzed separately, as EAR is most common during the first few weeks after LT, generally preceding CMV infection.

Table 1. Demographic and baseline clinical characteristics of patients included in the retrospective analysis

Variable	Total n=639	R- D- n=127	R- D$^+$ n=122	R$^+$ n=390	P-value
Recipient age, years	50 (16-71)	47 (17-68)	48 (16-71)	51 (16-67)	0.121
Recipient, female	264 (41%)	46 (36%)	54 (44%)	164 (42%)	0.039
Recipient BMI, kg/m²	25 (16-43)	24 (17-39)	24 (17-37)	25 (16-43)	0.299
Primary liver diseases					< 0.001
AHF	119 (19%)	23 (18%)	25 (20%)	71 (18%)	
HCC	64 (10%)	10 (8%)	10 (8%)	44 (11%)	
PBC/PSC/AIH	149 (23%)	40 (31%)	37 (30%)	72 (18%)	
HBV/HCV	84 (13%)	5 (4%)	2 (2%)	77 (20%)	
Alcoholic cirrhosis	71 (11%)	14 (11%)	17 (14%)	40 (10%)	
Cryptogenic cirrhosis	41 (6%)	10 (8%)	8 (7%)	23 (6%)	
Others	111 (17%)	25 (20%)	23 (19%)	63 (16%)	
Donor age, years	46 (8-78)	43 (13-73)	45 (12-72)	46 (8-78)	0.149
Donor, female	333 (52%)	49 (39%)	61 (50%)	223 (57%)	0.001
DCD donor	49 (8%)	8 (6%)	9 (7%)	32 (8%)	0.775
Cold ischemia time, minutes	470 (114-1099)	476 (120-1099)	479 (114-988)	455 (133-913)	0.441
Warm ischemia time, minutes	36 (14-143)	34 (14-106)	35 (17-143)	37 (16-129)	0.121
Re-LT	75 (12%)	15 (12%)	9 (7%)	51 (13%)	0.233
Basiliximab as induction immunosuppression	375 (59%)	82 (65%)	72 (59%)	221 (57%)	0.290
Calcineurin inhibitor					0.645
Cyclosporin A	249 (39%)	55 (43%)	46 (38%)	148 (38%)	
Tacrolimus	367 (57%)	70 (55%)	68 (58%)	229 (59%)	
CMV prophylaxis	104 (16%)	-	104 (85%)	-	< 0.001
CMV viremia	145 (23%)	4 (3%)	59 (48%)	82 (21%)	< 0.001
Timing of CMV viremia detection after LT, days	35 (2-2502)	30 (17-35)	45 (22-288)	31 (2-2502)	0.001
Timing of first CMV viremia after LT < 180 days	134 (92%)	4 (100%)	54 (92%)	76 (93%)	0.703
Peak CMV-DNA copy number, copies/mL*	2.8 (1.8-6.8)	5.0 (4.0-6.8)	2.7 (1.8-5.3)	2.8 (1.9-6.5)	0.031
CMV IgG seroconversion**	97 (20%)	14 (14%)	83 (86%)	-	< 0.001
Early acute rejection	144 (23%)	26 (20%)	29 (24%)	89 (23%)	0.805
Timing of early acute rejection, days	9 (2-166)	8 (2-155)	9 (3-147)	8 (3-166)	0.999
Late acute rejection	41 (6%)	11 (9%)	2 (2%)	28 (7%)	0.048
Timing of late acute rejection, days	487 (186-6368)	997 (208-2967)	353 (186-521)	379 (206-6368)	0.138

* Log_{10} transformed

** Only patients with follow-up ≥ 180 days were taken into account (R-D- n=101, R-D$^+$ n=97, R$^+$ n=299; total n=497), and from 9 patients serology data after LT were not available (R-D- n=5, R-D$^+$ n=4).

Statistical analysis

Patient baseline characteristics were summarized using median with range for continuous variables and percentage for discrete variables. Differences between groups were compared by Pearson Chi-Square test or one-way Kruskal-Wallis test. Experimental data were analyzed using nonparametric Mann-Whitney U test (unpaired) or Wilcoxon matched pairs test (paired) when comparing two groups, and one-way Kruskal-Wallis test (unpaired) or Friedman test (paired) with Dunn's multiple comparison test when comparing three groups. In the retrospective analysis, EAR and LAR were used as separate endpoints. The cumulative incidences of EAR and LAR were estimated using the Kaplan-Meier method with log-rank test. Analysis of risk factors for EAR and LAR was performed using Cox proportional-hazards regression model with likelihood ratio test. We first performed univariate analysis for each potential independent variable. Independent variables with P-values less than 0.2 were included in the multivariate analysis together with CMV serostatus and viremia. Linearity of continuous variables and clinical relevant interactions were tested. Where multiple pairwise comparisons were made, a Bonferroni correction on the alpha level was applied. SPSS v.21 was used for statistical analysis, and P-values < 0.05 were considered significant.

RESULTS

CMV-positivity is associated with the expansion of effector memory T-cell subsets in peripheral blood after liver transplantation

We prospectively collected PBMC samples from 34 patients with distinct CMV status before, at 1 month and at 6 months after LT. Peripheral T-cell subsets were analyzed by flow cytometry on basis of CCR7 and CD45RO expression (Figure 1A)(10). Patients were grouped based on CMV status (R-D- n=7, R-D$^+$ n=10, R$^+$ n=17), and distribution of CD8$^+$ (Figure 1B) and CD4$^+$ (Figure 1C) T-cell subsets at three time points were compared.

The proportion of both CD4$^+$ and CD8$^+$ T-cell subsets remained stable in CMV-negative patients (R-D-). However in CMV primary infection (R-D$^+$) and R$^+$ patients, the proportion of CD8$^+$ TNaive and TCM decreased continuously within the first 6 months, while the percentages of CD8+ TEM and TEMRA increased. Particularly CD8$^+$ T-cell subsets from R-D$^+$ patients underwent the most dramatic changes, characterized by rapidly increasing percentage of TEMRA (pre-LT 9%, 6 months 62%; median values). When dividing all patients at increased risk for CMV-replication (R-D$^+$ and R$^+$) by proven viremia, increasing percentage of CD8$^+$ TEMRA were observed in both groups (Supplemental Figure 2), suggesting that the TEMRA increase was a result of CMV-positivity, rather than CMV-replication. However it was likely that not all viremia episodes were detected in R+ patients. The change in CD8$^+$ T-cell subset distribution was a manifestation of ef-

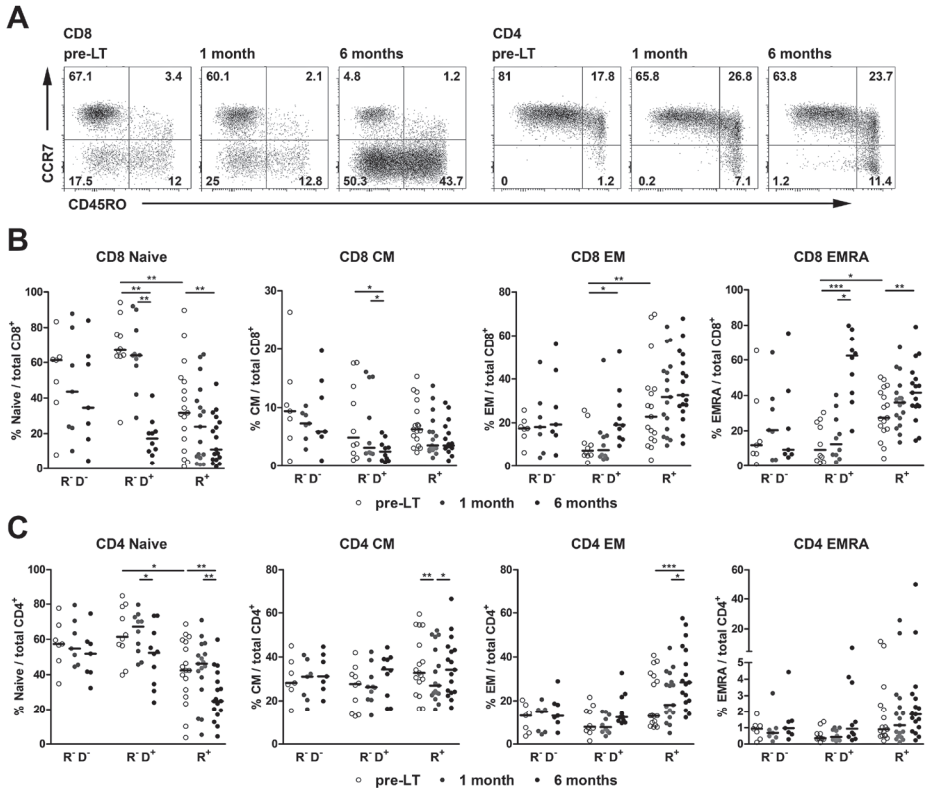

Figure 1.
Changes of circulating $CD4^+$ and $CD8^+$ T-cell subsets after LT in relation to patient CMV status. (A) T-cell subsets were defined as T_{Naive} ($CCR7^+CD45RO^-$), T_{CM} ($CCR7^+CD45RO^+$), T_{EM} ($CCR7^-CD45RO^+$), and T_{EMRA} ($CCR7^-CD45RO^-$), shown as representative FACS plots from one patient. Patients were grouped based on CMV status (R-D- n=7, $R\text{-}D^+$ n=10, R^+ n=17). Distribution of (B) $CD8^+$ and (C) $CD4^+$ T-cell subsets before (white), at 1 month (grey) and at 6 months (black) after LT were compared. Horizontal lines indicate mean values. *P<0.05, **P<0.01, ***P<0.001.

fector memory T-cell expansion rather than a selective disappearance of naive T cells, as absolute numbers of TEM and TEMRA increased in $R\text{-}D^+$ and R^+ patients, while the absolute numbers of TNaive remained relatively stable (Supplemental Figure 3). As for the $CD4^+$ compartment, the percentages of TNaive also decreased within 6 months in CMV primary infection and R^+ patients, which was mainly compensated by an increased percentage of TEM. Together, these data show that CMV-positivity is associated with the expansion of effector memory T-cell subsets in peripheral blood after LT.

CD8$^+$ T cells from CMV primary infection patients develop donor-specific hyporesponsiveness

We hypothesized that the expansion of effector memory T-cell subsets driven by CMV might increase the frequencies of alloreactive T cells, as cross-reactive viral-specific memory T cells are common (15). Thus we quantified the frequencies of donor-specific and third party-reactive T cells in 51 patients at minimum 6 months after LT by measuring the allogeneic activation-induced CD137 expression (Supplemental Figure 1A, B). The numbers of donor-recipient and third party-recipient HLA-mismatches were similar between groups (Supplemental Figure 4).

Overall CD8$^+$ T cells showed donor-specific hyporesponsiveness (Figure 2A, left panel). We did not observe higher frequencies of alloreactive T cells in CMV primary infection or R$^+$ patients than in CMV-negative patients. Unexpectedly, patients with primary infection showed prominent donor-specific hyporesponsiveness in CD8$^+$ T cells (Figure 2B, left panel), which was evident in TNaive, TCM and TEM (Figure 2C). In contrast, no donor-specific hyporesponsiveness was observed in CMV-negative patients, while in R$^+$ patients significant donor-specific hyporesponsiveness was only observed in TEM. CD4$^+$ T cells did not show any significant donor-specific hyporesponsiveness (Figure 2A, right panel), and the frequencies of alloreactive CD4$^+$ T cells were also similar in patients with distinct CMV status (Figure 2B, right panel).

When focusing on patients with proven viremia, donor-specific hyporesponsiveness was only seen in R-D$^+$ patients, and the frequency of donor-specific CD8$^+$ T cells in R-D$^+$ patients were lower than that in R$^+$ patients (Supplemental Figure 5), indicating that the donor-specific hyporesponsiveness was not due to CMV-replication as such. However, the peak CMV-DNA copy number tended to be negatively associated with the frequency of donor-specific CD8$^+$ T cells (P=0.06), but not with third-party reactive CD8$^+$ T cells or alloreactive CD4$^+$ T cells (Supplemental Figure 6).

Furthermore, we assessed the subset composition of alloreactive CD8$^+$ T cells (CD8$^+$CD137$^+$ T cells) (Supplemental Figure 1B). Compared to CMV-negative and R$^+$ patients, donor-specific T cells from patients with CMV primary infection were predominantly TEMRA (R-D- 29.7%, R-D$^+$ 57.7%, R$^+$ 33.1%; mean values) (Figure 2D, E), which is possibly due to the robust donor-specific hyporesponsiveness in other subsets except for TEMRA.

CD8$^+$ TEMRA are a minor T-cell population infiltrating liver allografts

As TEMRA were the major CD8$^+$ T-cell subset present in peripheral blood after LT and comprised the majority of residual donor-specific CD8$^+$ T cells in patients with CMV primary infection, we wondered whether CD8$^+$ TEMRA are abundantly present in liver allografts and thereby contribute to allograft rejection. To investigate this, we isolated intrahepatic lymphocytes (IHLs) from liver allografts which were explanted during re-LT

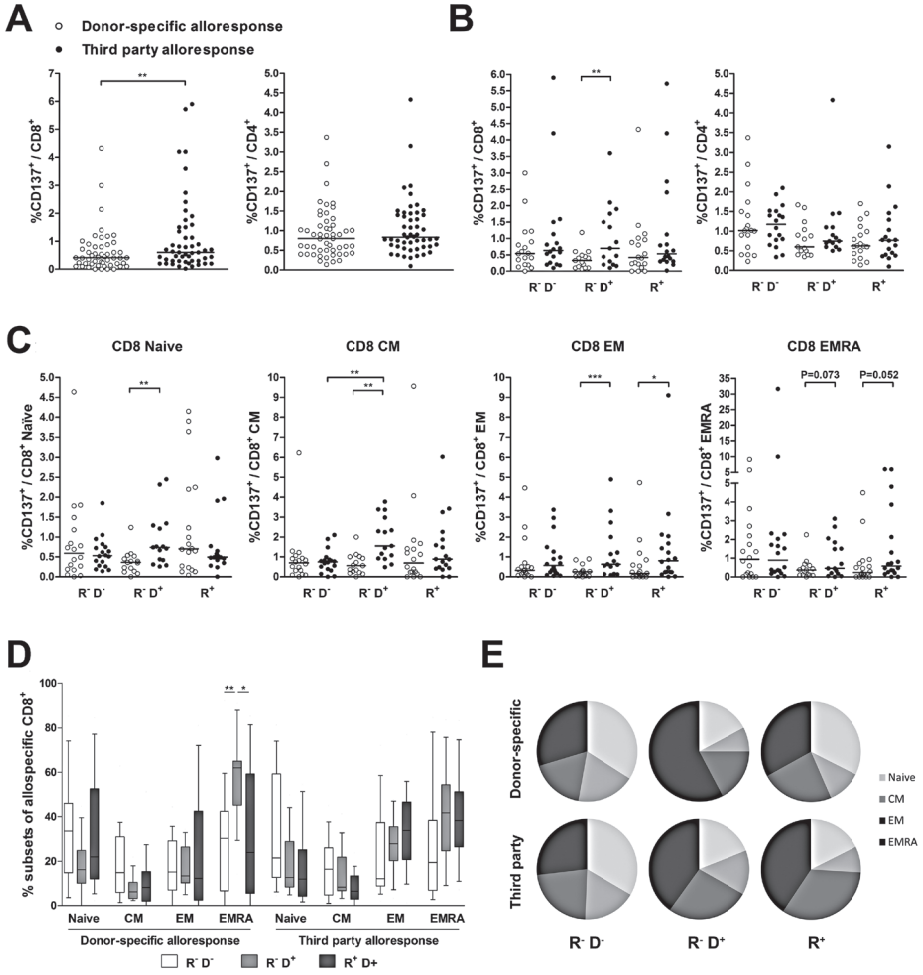

Figure 2.

CMV primary infection is associated with the development of donor-specific $CD8^+$ T-cell hyporesponsiveness after LT. The frequencies of peripheral alloreactive $CD8^+$ and $CD4^+$ T cells were analyzed by flow cytometric determination of CD137 expression on T cells after allogeneic stimulation. (A) Overall frequencies of donor-specific and third party-reactive $CD8^+$ (left panel) and $CD4^+$ (right panel) T cells were compared (n=51). Alloreactive T-cell frequencies of (B) total $CD8^+$ (left panel) and $CD4^+$ (right panel) T cells, and of each (C) $CD8^+$ T-cell subset were compared between patients with different CMV status (R-D- n=18, R-D^+ n=15, R^+ n=18). Horizontal lines indicate median values. Subset composition of alloreactive $CD8^+$ T cells was assessed by measuring CD45RO and CCR7 expression on $CD137^+CD8^+$ T cells. (D) Proportions of each T-cell subset within donor-specific and third party-reactive $CD8^+$ T cells are shown as median values and interquartile ranges, (E) and summarized as pie charts presenting mean proportions. *P<0.05, **P<0.01, ***P<0.001.

(n=10; Supplemental Table 2). IHLs isolated from heathy donor livers prior to LT were used as healthy control. IHLs contained mainly CD4$^+$ and CD8$^+$ TEM and hardly any TNaive and TCM (Figure 3A, B). CD8$^+$ TEMRA were present in the liver allografts but accounted for a significantly smaller proportion than in paired PBMC samples and IHLs of healthy donor livers (Figure 3B). The ratio of CD8$^+$ TEM and TEMRA in liver allografts was 5-fold

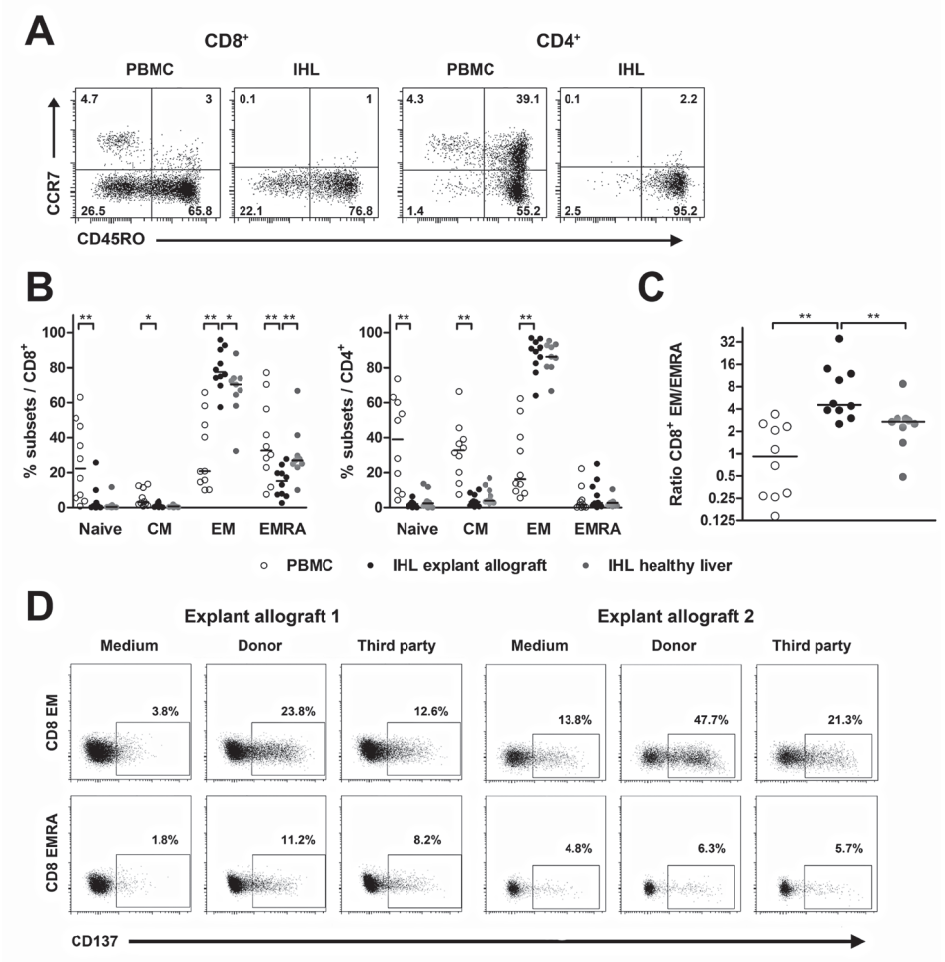

Figure 3.
CD8$^+$ TEMRA are a minor T-cell population infiltrating liver allografts. Intrahepatic lymphocytes (IHLs) were isolated from explant liver allografts to study the subset composition of graft infiltrating T cells. CD8$^+$ and CD4$^+$ T-cell subsets of IHLs of explant allografts (n=10) were compared to those of paired PBMCs, and IHLs of healthy donor livers (n=9), and are shown (A) as representative FACS plots (PBMCs and paired explant IHLs) from one patient and (B) are summarized. (C) Ratios CD8$^+$ TEM and TEMRA were also compared between groups. Large amounts of IHLs were isolated from two explant allografts and were co-cultured with donor and third-party splenocytes. Donor-specific and third party-reactive T cells were identified by CD137 upregulation. (D) Results are shown by FACS plots and percentages of CD137$^+$ T cells for each subset are depicted in the plots for both allografts. *P<0.05, **P<0.01.

higher than that in peripheral blood, and 1.7-fold higher than that in healthy donor livers (median values, Figure 3C). Explant allografts with AR activity tended to have lower percentages of CD8$^+$ TEMRA and a higher CD8$^+$TEM/TEMRA ratio (Supplemental Figure 7). Sufficient amounts of IHLs were isolated from two of the liver explants to measure their allogeneic responses (Figure 3D). CD8$^+$ TEM were enriched for donor-specific T cells, as 20% and 33.9% of CD8$^+$ TEM from these two IHLs samples, respectively, were reactive to donor splenocytes, while 8.8% and 7.5% of them responded to third-party stimulation (CD137 expression in conditions without stimulation were subtracted). In contrast, CD8$^+$ TEMRA contained less alloreactive cells than TEM. Altogether, these data indicate that TEM preferentially infiltrate liver allografts, while CD8$^+$ TEMRA largely remain in the circulation.

CMV R-D+ status is associated with the protection against late acute rejection

To study whether the donor-specific T-cell hyporesponsiveness that we observed in CMV primary infection patients has any clinical impact, we performed a retrospective study on 639 patients that underwent LT in our center between 1992 and 2010 to investigate the impact of CMV infection on EAR and LAR. One, 3 or 6 months have been variably used in literature as the cut-off to define EAR and LAR (21). The first episodes of CMV viremia were detected at median 35 days after LT, 92% of which were within the first 6

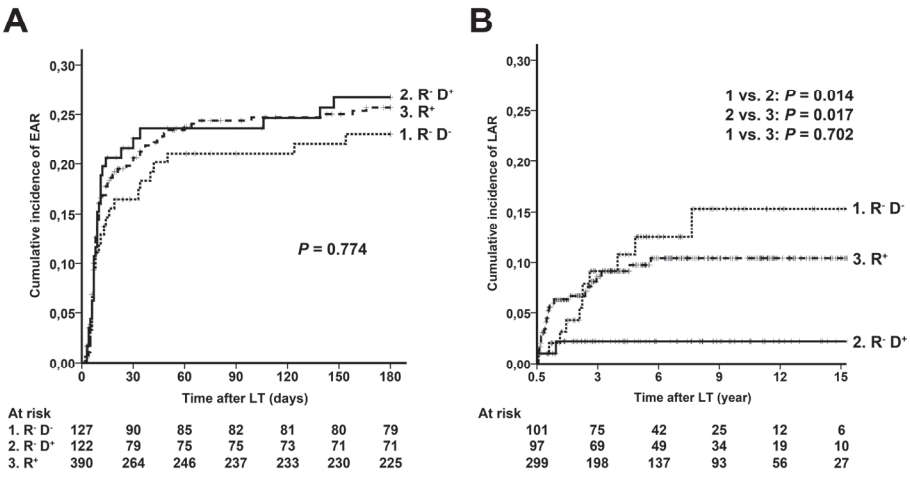

Figure 4.
CMV R-D$^+$ status is associated with the protection against late acute rejection. Patients were grouped based on CMV serostatus before LT as follows: Group 1: R-D-; Group 2: R-D$^+$; Group 3: R$^+$. The cumulative incidences of (A) early acute rejection (EAR) and (B) late acute rejection (LAR) were estimated using the Kaplan-Meier method and differences between curves were analyzed using the log-rank test. The number of patients at risk are depicted below the graphs.

months (Table 1). Thus we choose 6 months as the cut-off to define LAR in order to focus on the effect of CMV infection on graft rejection and not the other way around. Of the 639 patients, 144 (22.5%) developed EAR (median 9 days; range 2-166 days), and 41 (6.4%) developed LAR (median 487 days; range 186-6368 days) (Table 1).

EAR and LAR were set as separate endpoints for risk factor analysis. CMV serostatus was not associated with the cumulative incidence of EAR (P=0.77) (Figure 4A), while the incidence of LAR in R-D$^+$ patients was lower than in R-D- patients (P=0.014) and R$^+$ patients (P=0.017) (Figure 4B). In univariate Cox regression analysis, recipient age (P=0.007), recipient BMI (P=0.026), female donor (P=0.034), warm ischemia time (P<0.001), basiliximab induction (P<0.001), tacrolimus as CNI (P<0.001), and CMV viremia (P=0.008) were

Table 2. Risk factor analysis for late acute rejection following liver transplantation

	Univariate analysis			Multivariate analysis		
Variable	**HR**	**95% CI**	**P Value**	**HR**	**95% CI**	**P Value**
Recipient age, year	0.99	(0.97-1.02)	0.455			
Recipient, female	1.29	(0.70-2.39)	0.416			
Recipient BMI, kg/m²	1.04	(0.97-1.12)	0.309			
Primary liver disease			0.189			0.217
HBV/HCV (Ref)	1.00			1.00		
AHF	1.45	(0.49-4.34)		1.75	(0.57-5.44)	
HCC	0.29	(0.03-2.50)		0.35	(0.04-2.99)	
PBC/PSC/AIH	1.54	(0.56-4.29)		1.90	(0.67-5.43)	
Alcoholic cirrhosis	0.49	(0.10-2.54)		0.61	(0.12-3.16)	
Cryptogenic cirrhosis	2.04	(0.59-7.07)		2.22	(0.63-7.87)	
Others	0.81	(0.24-2.81)		1.05	(0.30-3.72)	
Donor age, year	0.99	(0.97-1.01)	0.239			
Donor, female	1.35	(0.72-2.51)	0.342			
DCD donor	1.43	(0.44-4.65)	0.575			
Cold ischemia time, 10 min	0.99	(0.97-1.01)	0.344			
Warm ischemia time, 10 min	1.01	(0.89-1.15)	0.877			
Re-LT	0.94	(0.33-2.63)	0.900			
Basiliximab induction	0.97	(0.51-1.85)	0.920			
Calcineurin inhibitor, Tacrolimus	0.63	(0.34-1.19)	0.154	0.69	(0.36-1.31)	0.258
Early acute rejection	0.90	(0.44-1.86)	0.781			
CMV serostatus			0.015			0.015
R- D- (Ref)	1.00			1.00		
R- D$^+$	0.18	(0.04-0.81)		0.18	(0.04-0.86)	
R$^+$	0.87	(0.43-1.76)		0.99	(0.47-2.05)	
CMV viremia	0.87	(0.42-1.83)	0.711	1.10	(0.50-2.43)	0.813
Peak copy number during viremia	1.07	(0.55-2.09)	0.846			

significantly associated with EAR (Supplemental Table 3). Meanwhile, CMV serostatus (P=0.015) was the only factor associated with LAR (Table 2).

In the multivariate Cox regression analysis, recipient age (P=0.046, hazard ratio [HR]=0.99), female donor (P=0.020, HR=0.67), basiliximab induction (P=0.015, HR=0.59), tacrolimus as CNI (P<0.001, HR=0.47), and CMV viremia (P=0.018, HR=1.62) were considered as independent factors associated with EAR (Supplemental Table 3). In contrast, CMV serostatus was the only independent factor associated with LAR (P=0.015; HR for R-D$^+$=0.18; HR for R$^+$=0.99) (Table 2). CMV seroconversion and the use of CMV prophylaxis were not included in the analysis as they largely overlapped with R-D$^+$ status, and yielded the same association as R-D$^+$ status (data not shown). Altogether, these data indicate that CMV primary infection after LT protects patients against the occurrence of LAR, corroborating the *ex vivo* data on donor-specific T-cell hyporesponsiveness that we observed in CMV primary infection patients.

CMV primary infection patients show the highest Vδ1/Vδ2 γδ T-cell ratio after LT

To further investigate whether patients with CMV primary infection show signs of tolerance, we measured peripheral Vδ1 and Vδ2 γδ T-cell subsets in patients at minimum 6 months after LT, and calculated the Vδ1/Vδ2 γδ T-cell ratio, which has been shown to be associated with operational tolerance after LT (16-18). CMV primary infection patients contained the highest percentage of Vδ1 γδ T cells within peripheral CD3$^+$ cells, while CMV-negative patients contained the lowest (R-D- 0.73%, R-D$^+$ 5.36%, R$^+$ 2.29%; median values) (Figure 5A). In contrast, the percentages of Vδ2 γδ T cells were similar (R-D- 0.56%, R-D$^+$ 0.27%, R$^+$ 0.39%; median values) (Figure 5B). As a result, patients with CMV primary infection showed higher Vδ1/Vδ2 γδ T-cell ratio than the other two groups (R-D- 1.39, R-D$^+$ 10.81, R$^+$ 6.31; median values) (Figure 5C).

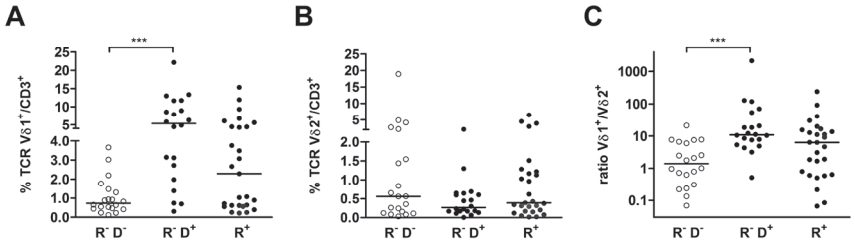

Figure 5.
CMV primary infection patients show the highest peripheral Vδ1/ Vδ2 γδ T cell ratio after LT. Peripheral blood Vδ1 and Vδ2 γδ T-cell subsets were analyzed by flow cytometry in LT patients. The percentages of (A) Vδ1 and (B) Vδ2 γδ T cells within total CD3$^+$ T cells, and (C) the subsequent Vδ1/ Vδ2 γδ T-cell ratio were compared between patients with different CMV status (R-D- n=20, R-D$^+$ n=20, R$^+$ n=27). Horizontal lines indicate median values. ***P<0.001.

DISCUSSION

Immune responses resulting from viral infections are proposed to promote allograft rejection and prevent the establishment of tolerance (14). Cross-reactive viral-specific memory T cells are common in humans. Approximately 45% of viral-specific, including CMV-specific, T-cell clones are cross-reactive to at least one allogeneic HLA molecule (15). Unexpectedly, and contrary to our initial hypothesis, we did not find higher frequencies of alloreactive T cells in either R+ patients or patients who developed CMV primary infection. In contrast, we found a robust donor-specific $CD8^+$ T-cell hyporesponsiveness in patients with CMV primary infection. In accordance with the donor-specific hyporesponsiveness, retrospective analysis showed that LT patients with R-D+ serostatus had a significantly lower risk to develop LAR. Moreover, CMV primary infection patients showed the highest ratio of peripheral Vδ1/Vδ2 γδ T cells, which has shown to be associated with operational tolerance after LT. This is the first study showing that CMV primary infection remarkably reduces donor-specific T-cell reactivity and is a protective factor against the occurrence of LAR, suggesting a prominent role for CMV infection in transplant tolerance to liver allografts in humans.

We first found that CMV-positivity, particularly CMV primary infection, is associated with the expansion of $CD8^+$ effector memory T-cell subsets in peripheral blood after LT. Since TEM and TEMRA subsets can mount rapid effector responses upon allostimulation (8), they are hypothesized to be detrimental to allografts (22). Nonetheless we do not support a detrimental role of $CD8^+$ TEMRA for LT patients, as our data suggest that $CD8^+$ TEMRA rarely infiltrate liver allograft but largely remain in circulation. A recent study shows that increased numbers of circulating $CD8^+$ TEMRA before kidney transplantation are associated with a reduced incidence of AR (23). Similarly, few $CD8^+$ TEMRA were found in rejecting kidney allografts (24). However we cannot rule out the possibility that TEMRA may change their phenotype into TEM upon infiltration.

An intriguing question is how CMV infection induces donor-specific T-cell hyporesponsiveness. CMV infection is known to drive immunosenescence, which has been suggested to promote kidney allograft acceptance in elderly recipients (25). Immunosenescence driven by CMV is manifested by inflation of CMV-specific effector memory T cells. It has been postulated that the massively expanded CMV-specific effector memory T-cell pool competes with newly generated T cells for niches and survival factors, and as a consequence T-cell diversity and responses to other pathogens are restricted (26, 27). Indeed both in humans and mice CMV infection causes impaired T-cell immunity to other pathogens (28, 29), and CMV infection after organ transplantation is associated with a higher incidence of opportunistic infections (30). This is supported by previous findings showing that high numbers of CMV-IE-1-specific memory T cells are associated with lower numbers of alloreactive T cells and improved renal allograft function after

kidney transplantation (31), and that high CMV-specific $CD4^+$ T-cell responses correlate with protection from cardiac allograft rejection (32). We hypothesize that the donor-specific T-cell hyporesponsiveness observed in CMV primary infection patients might be related to the multifaceted immune evasion capacity used by CMV to establish latency, in particular its capacity to modulate antigen presentation (33). Alexander et al. reported the development of hematopoietic chimerism and donor-specific hyporesponsiveness in a patient with severe CMV disease early after LT (34). We also reported three cases of long-term hematopoietic chimerism within liver allografts (35), and interestingly all three patients were R^+D^+ with detection of viremia in two of them (unpublished data). The immune-modifying effects of CMV may have contributed to the engraftment of donor cells, leading to subsequent donor-specific hyporesponsiveness. Moreover, dendritic cells present in liver graft are the main instigators of T-cell immunity against the graft (36, 37), but CMV infected dendritic cells are impaired in their ability to stimulate allogeneic lymphocytes (38). The immunomodulatory effect of primary infection has also been suggested, shown by higher bacterial and fungal infection-related mortality in $R\text{-}D^+$ patients after allogeneic hematopoietic stem cell transplantation, which is independent from CMV-replication, diseases and treatments (39). We hypothesize that the rapidly inflated anti-CMV immunity following primary infection in an immunocompromised environment, may contribute to its immunomodulatory effect. The exact mechanisms attributing to this phenomenon remain to be investigated.

In the retrospective analysis, we found that CMV viremia was positively associated with EAR. However, EAR occurred on median 9 days after LT, preceding the detection of CMV viremia in general. This finding is in line with the hypothesis that alloimmune stimulation triggers CMV-replication from latency (40). Heavy immunosuppression during EAR treatment may also increase the risk of CMV viremia. In the multivariate analysis older patient age decreased the risk of EAR, which could be explained by the senescence of immune system (25, 41). The use of basiliximab induction and tacrolimus both decreased the risk of EAR, which is in line with previous findings (42, 43). Patients with female donors had a decreased incidence of EAR. However this could be a finding by chance, as previous studies showed that donor gender does not influence AR incidences. In contrast, we found that CMV $R\text{-}D^+$ serostatus was an independent protective factor against LAR. Despite antiviral prophylaxis, the rate of seroconversion in $R\text{-}D^+$ patients was 86%, in agreement with previous study (44), indicating that almost all $R\text{-}D^+$ patients get primarily infected eventually. LAR was not associated with viremia or peak CMV-DNA copy number, suggesting that the lower incidence of LAR was not due to CMV-replication as such. We cannot exclude an effect of prophylaxis, however it is unlikely since higher dosages of valGCV or GCV were administrated in case of CMV viremia, but viremia was not associated with lower incidence of LAR. As there is no indication that in R-D- or R^+ patients immunosuppression was prescribed differently hence affecting the occurrence

of LAR, the lower incidence of LAR is probably a reflection of the pro-tolerogeneic status of the patients with CMV primary infection. The discrepancy of EAR and LAR in relation to CMV infection indicates that CMV during active replication or in a quiescent state may have differential effects on graft rejection. Careful distinguishing CMV primary infection from non-primary infection, and between EAR and LAR in our analysis, may be possible reasons why this association has never been reported before.

Immunosuppression can be completely discontinued in more than 40% of stable, adult LT patients (45, 46). Whether CMV infection plays a role in achieving operational tolerance has yet not been investigated. However, studies sought to identify biomarkers for operational tolerance have found expansion of peripheral Vδ1 γδ T cells, and an increased Vδ1/Vδ2 ratio in tolerant LT patients. Vδ1/Vδ2 ratio has even been used as a surrogate marker to predict operational tolerance (16-18). Interestingly, expansion of Vδ1 γδ T cells is also a feature that is observed upon CMV infection (47, 48), arguing in favour of a potential association between CMV infection and liver graft tolerance. In addition to previous findings, we found that patients with CMV primary infection showed the highest Vδ1/Vδ2 ratio. This finding corroborates the lower incidence of LAR in our retrospective analysis and suggests that these patients may have the highest chance to achieve operational tolerance after immunosuppression discontinuance. Since we do not minimize immunosuppression routinely, we are not able to demonstrate a direct link between CMV infection and operational tolerance.

There are limitations of this study that need to be acknowledged. First, we focused on allogeneic T-cell responses in LT patients with different CMV status, however anti-CMV responses were not studied, which could help understanding the interplay between allogeneic and anti-viral immune responses (31). Second, we were not able to demonstrate the direct link between the donor-specific T-cell hyporesponsiveness and the lower incidence of LAR in primary infection patients, as the overall incidence of LAR was low and we did not have enough PBMC samples available from LAR patients. A case-control study comparing LAR cases to patients with stable graft function, or a large-scale prospective study with long follow-up, may reveal the direct relationship between T-cell responses and LAR.

To conclude, the primary findings of this study are that patients with CMV primary infection after LT show donor-specific $CD8^+$ T-cell hyporesponsiveness, are protected from the occurrence of LAR, and show signs of operational tolerance. Further investigation into the role of CMV infection in the development of operational tolerance is necessary. Since CMV status is easily measured without additional effects or costs, this parameter can be taken into account by physicians when selecting patients for tapering or withdrawing immunosuppressive therapy in LT patients. Altogether, for the first time to our knowledge, we show evidences that CMV primary infection may promote immunological tolerance towards allogeneic liver graft in humans.

Acknowledgements

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Supplemental Table 1. Characteristics of patients included for peripheral blood T-cell analysis

Total: 75 patients	**R- D- (n=20)**	**R- D^+ (n=20)**	**R^+ (n=35)**	**P value**
Recipient				
Age (median, range), years	54 (33-65)	48 (21-64)	54 (19-68)	0.190
Sex, female	7 (35%)	10 (50%)	14 (40%)	0.614
Donor				
Age (median, range), years	48 (16-64)	58 (22-73)	51 (13-78)	0.118
Sex, female	8 (40%)	13 (65%)	15 (43%)	0.202
Primary liver disease*				0.831
AHF	1 (5%)	4 (20%)	4 (11%)	
HBV/HCV	1 (5%)	1 (5%)	3 (9%)	
HCC	3 (15%)	3 (15%)	9 (26%)	
Metabolic diseases	2 (10%)	1 (5%)	3 (9%)	
PSC/PBC/AIH	9 (45%)	6 (30%)	8 (23%)	
other	4 (20%)	5 (25%)	8 (23%)	
Initial immunosuppressive regimen				
Basiliximab	16 (80%)	19 (95%)	29 (83%)	0.347
Tacrolimus	20 (100%)	20 (100%)	33 (94%)	0.309
Mycophenolate mofetyl	16 (80%)	13 (65%)	25 (71%)	0.569
Early acute rejection	4 (20%)	3 (15%)	6 (17%)	0.916
Late acute rejection	1 (5%)	1 (5%)	1 (3%)	0.894
CMV viremia	0 (0%)	17 (85%)	10 (29%)	0.000
Timing of viremia (median, range), days	-	79 (23-288)	27 (12-60)	0.003
Peak CMV-DNA copy number, copies/mL**	-	2.9 (1.9-5.0)	2.5 (2.1-5.5)	0.628
CMV IgG seroconversion	0 (0%)	20 (100%)	-	0.000

*AHF, acute hepatic failure; HCC, hepatocellular carcinoma; HBV, hepatitis B virus infection; HCV, hepatitis C virus infection; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; AIH, autoimmune hepatitis.
**Log_{10} transformed

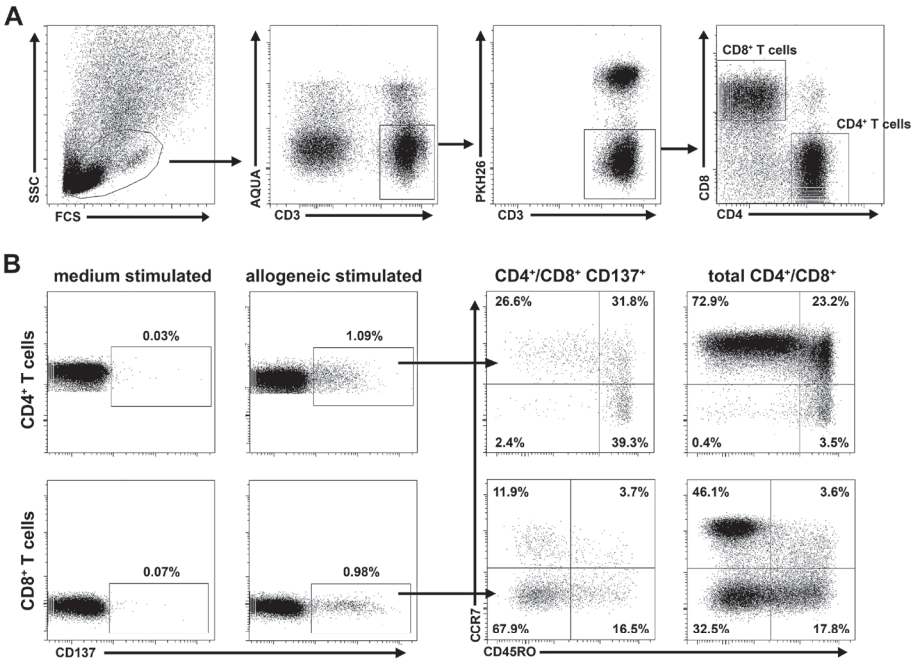
Supplemental Table 2. Characteristics of patients studied for liver allograft infiltrating T cells

Patient no.	Age	Sex	Time since first LT	Re-LT indication	RAI score	CMV status
1	66	M	7 months	Hepatic artery thrombosis and HCV recurrence	7	R^+ D-
2	40	M	6 years	PSC recurrence	4	R- D-
3	67	M	3 years	Chronic rejection	3-4	R^+ D^+
4	59	M	8 years	HCV recurrence	-	R^+ D-
5*	56	M	1 year	Chronic rejection	-	R^+ D^+
6	56	F	1 year	Ischemic cholangiopathy	-	R- D^+
7	38	M	24 years	Chronic rejection	4	R- D-
8*	29	M	6 years	Chronic rejection	-	R^+ D^+
9	56	M	2 years	Chronic rejection	3-4	R^+ D-
10	50	M	2 years	PSC recurrence	-	R^+ D-

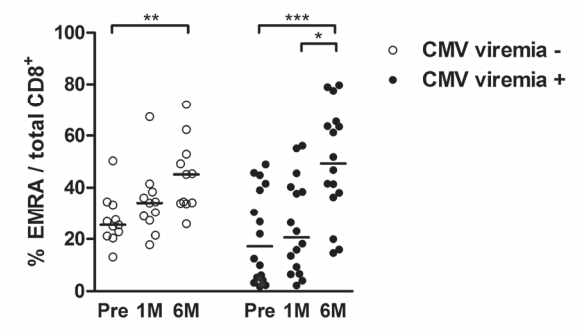
* Large amount of IHLs were isolated for functional experiments

Supplemental Table 3. Risk factor analysis for early acute rejection following liver transplantation

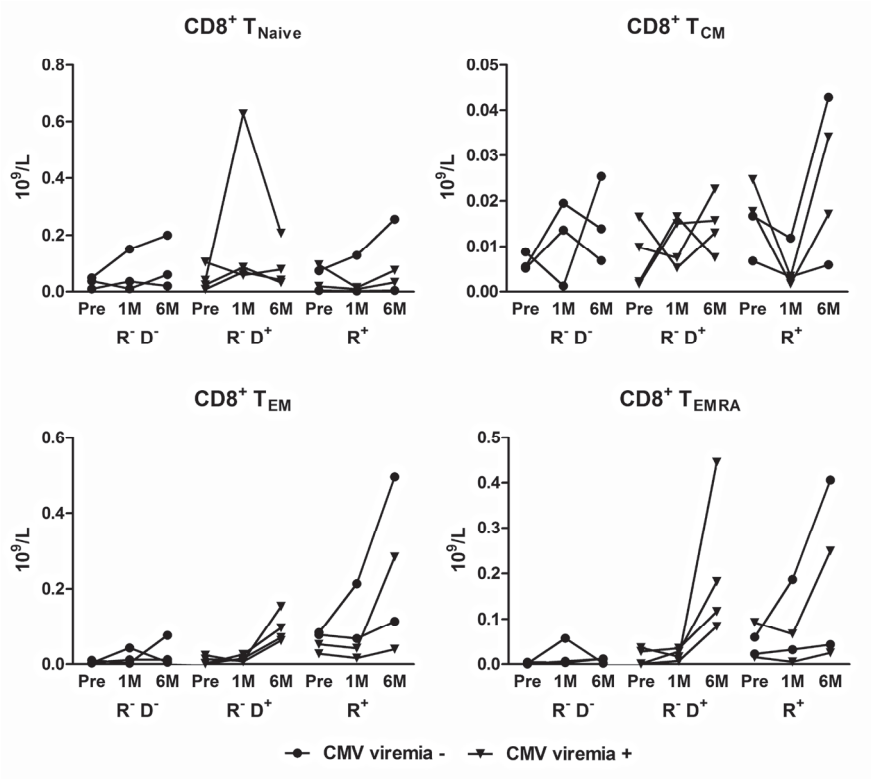
	Univariate analysis			Multivariate analysis		
Variable	**HR**	**95% CI**	**P Value**	**HR**	**95% CI**	**P Value**
Recipient age, year	0.98	(0.97-0.99)	0.007	0.99	(0.97-1.00)	0.046
Recipient, female	1.26	(0.91-1.76)	0.164	1.14	(0.81-1.60)	0.448
Recipient BMI, kg/m^2	0.96	(0.92-1.00)	0.026	0.98	(0.94-1.02)	0.375
Primary liver disease			0.415			
HBV/HCV (Ref)	1.00					
AHF	1.40	(0.78-2.53)				
HCC	0.95	(0.46-1.96)				
PBC/PSC/AIH	1.27	(0.72-2.24)				
Alcoholic cirrhosis	0.82	(0.39-1.72)				
Cryptogenic cirrhosis	0.64	(0.25-1.63)				
Others	1.10	(0.60-2.03)				
Donor age, year	0.99	(0.98-1.00)	0.192	1.00	(0.99-1.01)	0.904
Donor, female	0.70	(0.50-0.98)	0.034	0.67	(0.47-0.94)	0.020
DCD donor	0.50	(0.22-1.14)	0.065	0.91	(0.39-2.11)	0.820
Cold ischemia time, 10 min	1.01	(1.00-1.02)	0.067	1.00	(0.99-1.01)	0.354
Warm ischemia time, 10 min	1.19	(1.12-1.26)	0.000	1.05	(0.97-1.15)	0.249
Re-LT	0.85	(0.50-1.45)	0.535			
Basiliximab induction	0.39	(0.28-0.55)	0.000	0.59	(0.39-0.90)	0.015
Calcineurin inhibitor, Tacrolimus	0.36	(0.26-0.50)	0.000	0.47	(0.32-0.71)	0.000
CMV serostatus			0.773			0.856
R-/D- (Ref)	1.00			1.00		
R-/D$^+$	1.20	(0.71-2.04)		0.92	(0.51-1.67)	
R$^+$	1.14	(0.74-1.76)		1.05	(0.66-1.66)	
CMV viremia	1.62	(1.14-2.28)	0.008	1.62	(1.09-2.39)	0.018
Peak CMV-DNA copy number	1.17	(0.85-1.63)	0.352			



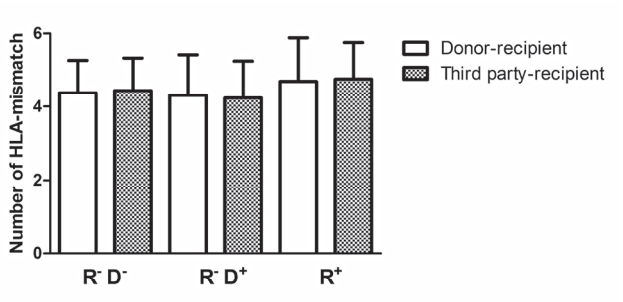
Supplemental Figure 1.
Determination of allogeneic activation-induced CD137 expression on $CD4^+$ and $CD8^+$ T cells to study the frequencies and subset composition of alloreactive T cells by flow cytometry. (A) AQUA staining was used to exclude dead cells, and stimulatory splenocytes were labeled by PKH26 and were excluded from the analysis. (B) Alloreactive $CD4^+$ and $CD8^+$ T cells were identified by the up-regulation of CD137 after 24 hours of co-culture. Subset composition of alloreactive T cells was analyzed by measuring the expression of CCR7 and CD45RO on $CD137^+$ T cells, and was defined as follows: TNaive ($CCR7^+CD45RO$-), TCM ($CCR7^+CD45RO^+$), TEM (CCR7-$CD45RO^+$), and TEMRA (CCR7-CD45RO-). All events in culture were recorded to ensure the detection of alloreactive T cells. Background expression of CD137 in conditions without allogeneic stimulation was subtracted during analysis.



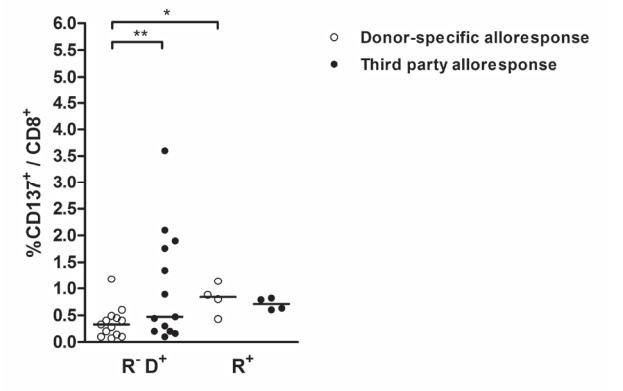
Supplemental Figure 2.
Comparison of CD8⁺ TEMRA changes in CMV-positive patients with or without proven viremia. Viremia was detected in 16 of 27 CMV-positive patients (R-D⁺ and R⁺). We compared the percentages of CD8⁺ TEMRA before (Pre), at 1 month (1M) and at 6 months (6M) after LT (one-way Friedman test) in both groups. Horizontal lines indicate median values. *P<0.05, **P<0.01, ***P<0.001.



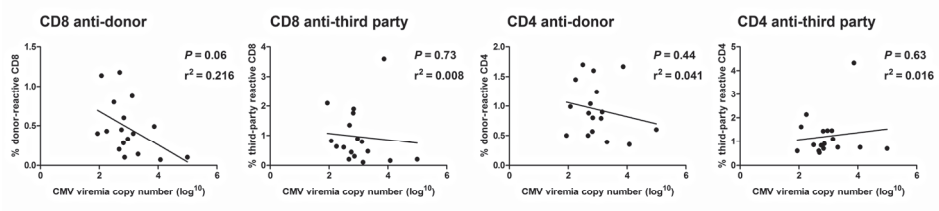
Supplemental Figure 3.
Changes in absolute numbers of CD8⁺ T-cell subsets after LT in relation to CMV status. Absolute numbers of CD8⁺ T-cell subsets were measured longitudinally in 11 patients (R-D- n=3, R-D⁺ n=4, R⁺ n=4) before (Pre), at 1 month (1M) and at 6 months (6M) after LT. Black dots represent patients without detected viremia, and black triangles represent patients with detected CMV viremia after LT. Absolute numbers are shown on the Y-axis.



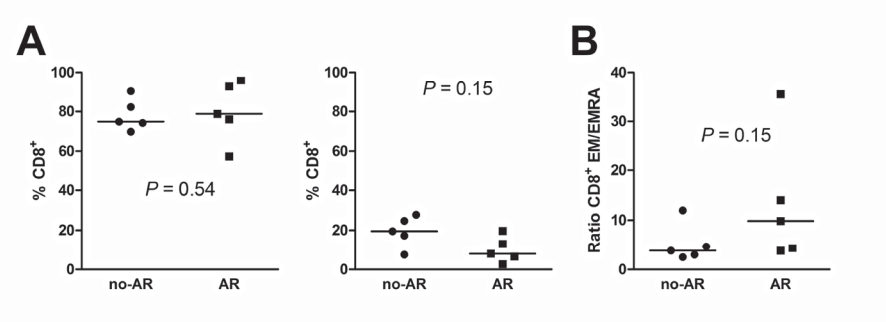
Supplemental Figure 4.
Numbers of HLA-mismatches between donor-recipient and third party-recipient in *ex vivo* allogeneic stimulations. The total numbers of HLA-mismatches at HLA-A, B, DR loci between donor-recipient and third party-recipient in the *ex vivo* allogeneic stimulation experiments are shown as mean with standard deviation, and were compared between the three experimental groups. No statistically significant difference was observed.



Supplemental Figure 5.
Alloreactive $CD8^+$ T-cell frequencies in patients with proven viremia. The frequencies of donor-reactive and third party-reactive $CD8^+$ T cells were compared between $R\text{-}D^+$ patients with viremia and R^+ patients with viremia. Horizontal lines indicate median values. *P<0.05, **P<0.01.



Supplemental Figure 6.
Correlation of alloreactive T-cell frequencies with CMV viremia level. In patients with proven viremia, the correlation between alloreactive T-cell frequencies and the peak CMV-DNA copy number during viremia was analyzed by linear regression. The CMV-DNA copy number was $\log_{10}$-transformed.



Supplemental Figure 7.
Comparison of CD8⁺ T-cell subsets in explant allografts with or without acute rejection activity. Five explant allografts presented acute rejection activity (AR, versus no-AR). (A) Percentages of intragraft CD8⁺ TEM, TEMRA, and (B) the subsequent ratio were compared (Mann-Whitney U test). Horizontal lines indicate median values.

SUPPLEMENTAL MATERIAL AND METHODS

Immunosuppression

The standard immunosuppressive therapy in our center consisted of prednisone, cyclosporine or tacrolimus, with or without azathioprine or mycophenolate mofetil (MMF). Since 1998, basiliximab was introduced as induction immunosuppression and was used in 58.7% of all patients in the retrospective cohort (Table 1), and meanwhile the use of cyclosporine was gradually replaced by tacrolimus. Tacrolimus was initiated within the first 5 days after transplantation in a dose of 1-2 mg/kg body weight/day. The target trough level was 10-15 ng/ml in the first month, 8-12 ng/ml between 1 to 6 months, 5-10 ng/ml between 6 to 12 months, and 4-8 ng/ml after 1 year. Cyclosporine was initiated within 24 hours post-reperfusion in a dose of 10-15 mg/kg body weight/day, and the dosage was adjusted to trough levels according to a range from 200-400 ng/mL during the first 3 months and thereafter 100-200 ng/mL. Acute rejection episodes were treated with high dose of methylprednisolone intravenously, and standard levels of immunosuppression were applied again after the rejection resolved. Immunosuppressive regimens were similar in patients from whom blood samples were collected, consisting of corticosteroids, MMF, tacrolimus and induction with basiliximab (Supplemental Table 1).

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll gradient centrifugation, and were cryopreserved for phenotyping and functional experiments. To isolate intrahepatic lymphocytes (IHLs), biopsies of explant allografts and pre-LT healthy donor livers were collected in University of Wisconsin (UW) preservation solution. Fresh tissue was cut into small pieces and digested with 0.5 mg/mL collagenase IV (Sigma-Aldrich, St. Louis, MO) and 0.1 mg/mL DNase I (Roche, Indianapolis, IN) for 30 minutes at 37°C. Cell suspensions were filtered through 70 µm cell strainers and IHLs were obtained by Ficoll density gradient centrifugation. Phenotyping and functional experiments were performed immediately after IHLs isolation.

Human splenocytes were isolated from splenic tissue derived from liver donors. Splenic samples were cut into small pieces and forced through 74 µm netwell filters (Costar, Corning International, NY) to obtain single cell suspensions. Mononuclear cells were isolated by standard Ficoll gradient centrifugation, and were cryopreserved for future experiments.

Flow cytometry and antibodies

The following antibodies were used: CD3-HorizonV500, CD4-APC-H7, CD8-Pacific Blue (BD Biosciences, San Diego, CA); TCR-Vδ1-FITC (Thermo Scientific, Waltham, MA); CD3-Pacific Blue, TCR-Vδ2-PE (BD Pharmingen, Erembodegem, Belgium); CD45RA-PE-Vio770

(Miltenyi Biotec, Bergisch Gladbach, Germany); CCR7-FITC (R&D System, Minneapolis, MN); CD45RO-PerCP-Cy5.5, CD137-APC (Biolegend, San Diego, CA); CD3-PE-Cy7, TCR-αβ-APC (eBioscience, San Diego, CA); CD3-FITC (Beckman Coulter, Marseille, France). Non-viable cells were excluded using 7-AAD (BD Biosciences, San Diego, CA) or LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Carlsbad, CA). Flow cytometry was performed on FACSCanto II flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed with BD FACSDiva software version 6.1.1.

Quantification of alloreactive T-cell frequencies

Alloreactive T cells were analyzed by determination of activation-induced CD137 expression on T cells, as previously described with minor modifications (1). In brief: $2.5x10^6$ PBMCs or intra-hepatic lymphocytes (IHLs) were co-cultured with donor or third-party (mismatched at HLA-A, B and DR loci with both donor and recipient) splenocytes at a 1:1 ratio in polypropylene tubes (BD Pharmingen, Erembodegem, Belgium) in duplicate, in 1 mL IMDM (Lonza, Breda, The Netherlands) supplemented with 10% heat-inactivated human serum and 1% Penicilline/Streptomycine. Co-stimulaton was provided by addition of 1 µg/mL anti-CD49d (BD Pharmingen, Erembodegem, Belgium) and 1 µg/mL anti-CD28 (Serotec, Kidlington, Oxford, UK). Prior to co-culture, the splenocytes were labeled with PKH26 (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions, to discriminate between stimulator splenocytes and responder PBMCs or IHLs during FACS analysis. As a control, cells were cultured in the presence of anti-CD49d and anti-CD28 mAb only, without allogeneic stimulation. After 24 hours, cells were harvested for FACS analysis. Alloreactive $CD4^+$ and $CD8^+$ T cells were identified by the upregulation of CD137 (Supplemental Figure 1). Background expression of CD137 in conditions without allogeneic stimulation was subtracted during analysis. This assay has been used in the identification and isolation of viral, tumor, and allospecific T cells regardless of their differentiation stage or cytokine production profile (1, 2).

CMV diagnostics and treatment strategies

CMV-DNA PCR measurements were only performed on a weekly basis in high risk patients ($R\text{-}D^+$) until 90 days post-LT. For all other patients, PCR was performed in case of clinical suspicion of CMV infection. Due to the non-protocolized CMV-DNA PCR monitoring, the incidence of CMV reactivation/reinfection in R^+ patients was probably underestimated. Low-dose (450 mg once daily) valganciclovir (valGCV) prophylaxis was administered to high risk patients, starting at day 7 and continuing up to day 90 post-LT. A therapeutic GCV-based regime, either intravenous GCV 5 mg/kg twice daily or valGCV 900 mg twice daily, was given to patients with positive PCR results. The regimen was given for 10 to 14 days, and PCR results were negative before withdrawing GCV therapy. Adjustments of

immunosuppressive therapy were made if necessary, and normal levels of immunosuppression were applied again after the clearance of viremia.

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CHAPTER 6

CMV-induced expression of CD244 after liver transplantation is associated with CD8$^+$ T-cell hyporesponsiveness to allo-antigen

E.L.D. de Mare-Bredemeijer[1]
X-L. Shi[1]
S. Mancham[1]
R. van Gent[1]
S.A. van der Heide-Mulder[1]
R. de Boer[2]
M.H.M. Heemskerk[2]
J. de Jonge[3]
L.J.W. van der Laan[3]
H.J. Metselaar[1]
J. Kwekkeboom[1]

1 Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam
2 Department of Hematology, Leiden University Medical Center, The Netherlands
3 Department of Surgery, Erasmus MC-University Medical Center, Rotterdam, The Netherlands

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ABSTRACT

Chronic presence of viral antigens can induce T-cell exhaustion, characterized by upregulation of co-inhibitory receptors and loss of T-cell function. We studied whether a similar phenomenon occurs after liver transplantation (LTx), when there is continuous exposure to allo-antigen. Expression of co-inhibitory receptors on circulating $CD4^+$ and $CD8^+$ T cells was longitudinally analyzed in 19 patients until 6 months after LTx, and cross-sectionally in 38 patients late (1-12 years) after LTx. Expression of the co-inhibitory receptors CD160 and CD244 on circulating $CD8^+$ T cells was already 6 months after LTx higher than pre-LTx, and the elevated expression was sustained late after LTx, with CD244 showing the most prominent increase. The strongest upregulation of CD244 on circulating $CD8^+$ T cells was observed in patients who experienced cytomegalovirus (CMV) infection after LTx. CMV infection was also associated with reduced $CD8^+$ T-cell proliferation and cytotoxic degranulation in response to allo-antigen late after LTx. Purified $CD244^+$ $CD8^+$ T cells of LTx patients showed lower proliferative responses to allo-antigen as well as to polyclonal stimulation than their CD244- counterparts. In addition, the $CD244^+$ $CD8^+$ T-cell population contained the majority of CMV-peptide-loaded MHC class I tetramer-binding cells. In conclusion, CMV infection after LTx, rather than persistence of allo-antigen, induces accumulation of dysfunctional $CD8^+CD244^+$ T cells in the circulation which persist on long-term, resulting in reduced frequencies of circulating allo-reactive $CD8^+$ T cells. These results suggest that CMV infection restrains $CD8^+$ T-cell allo-responses after LTx.

INTRODUCTION

After liver transplantation (LTx), most patients need lifelong immunosuppression to prevent rejection of the allograft, but some patients develop spontaneously immunological tolerance to their liver graft and can be completely withdrawn from all immunosuppression (1). This phenomenon occurs more frequently after LTx than in any other organ transplant setting (2), suggesting that the immunological alloresponse is skewed towards tolerance. Allograft rejection is mainly mediated by T cells of the recipient that respond to allogeneic donor antigens (3). In the transplantation setting, activation of allogeneic T cells via their T-cell receptor (TCR) is triggered by specific recognition of donor-derived allo-antigenic peptides presented by recipient major histocompatibility complex (MHC) molecules or by direct interaction of the TCR with allogeneic MHC molecules. However, T cells can also express co-stimulatory and co-inhibitory receptors that affect outcome of T-cell responses (4). Hence, these receptors might also influence the outcome of T-cell responses towards the allograft.

Expression of co-inhibitory receptors is upregulated on T cells in patients with chronic viral infections, such as hepatitis C virus (HCV) and HIV, and in patients with cancer (5-12). High expression of co-inhibitory receptors is associated with T-cell dysfunction, or "exhaustion", and in these patients virus-specific or tumor-specific T-cell responses are therefore impaired. T-cell exhaustion can be defined as a state of antigen-specific T-cell dysfunction in response to chronic persistence of high antigenic load (5). Exhausted T cells have poor proliferative and effector function, show sustained expression of co-inhibitory receptors and their transcriptional state is distinct from that of functional effector or memory T cells (5). Several co-inhibitory receptors have been described to be important in inhibiting T-cell responses, among which the most well-studied are: Programmed Death 1 (PD1), Lymphocyte-Activation Gene 3 (LAG3), T cell immunoglobulin mucin 3 (TIM3) , CD160 and CD244 (5, 6). PD1 has two ligands: PD-L1 and PD-L2 (7, 13). PD-L1 is expressed on both hematopoietic cells (mainly on dendritic cells (DC) and macrophages) and non-hematopoietic cells (parenchymal cells of many organs including liver; and endothelial cells) (7, 14). PD-L2 expression is restricted to DC and macrophages. Binding of PD1 to its ligand(s) negatively regulates T-cell responses (13, 15). LAG3 is a protein closely related to CD4, and mediates negative regulation of T-cell functions through interactions with its ligand MHC class II to which it binds with higher affinity than CD4 (16, 17). TIM3 inhibits $CD8^+$ T-cell responses by interaction with its ligand galectin-9 (8, 9). CD160 is a glycosylphosphatidylinositol-anchored receptor that inhibits T-cell responses upon binding to its ligand herpes virus entry mediator (HVEM) that is expressed on both hematopoietic and non-hematopoietic cells (parenchymal cells) (18). CD244, also called 2B4, can mediate both activating and inhibitory signals upon binding with its ligand CD48.

High levels of CD244 expression on T cells were found to be associated with inhibitory receptor function (10, 14, 19).

After organ transplantation, numbers of circulating T cells that react to donor allo-antigen decrease over time in a majority of patients (20-22). The mechanism underlying this phenomenon is as yet unclear. Whether chronic stimulation by the persistence of a high allo-antigenic load induces upregulation of co-inhibitory receptors and exhaustion of donor-specific T cells, as observed during chronic viral infections, is unknown. However, various experimental animal studies have shown enhanced rejection and/or decreased graft survival after blockade of co-inhibitory receptors in organ transplanted mice. This implies that co-inhibitory receptors are involved in suppressing allograft rejection in mice (23-26). However, the role of T-cell exhaustion and co-inhibitory receptor-ligand interactions in human solid organ transplantation has not yet been widely studied (25).

The aim of this study was to determine whether the expression of co-inhibitory receptors on circulating T cells is upregulated after LTx in humans, to analyze which clinical factors influence such upregulation, and to assess whether co-inhibitory receptors impair allogeneic T-cell responses after LTx. We hypothesized that long-term persistence of a high load of allo-antigens after LTx may induce exhaustion of allogeneic T-cells, characterized by upregulation of co-inhibitory receptors and hyporesponsiveness of $CD8^+$ T cells to allo-antigens.

MATERIALS AND METHODS

Study design and patients

Heparinized blood was collected at 1 and 6 months after transplantation from 19 primary liver transplant recipients who were transplanted in the Erasmus Medical Centre Rotterdam (The Netherlands) (early post-LTx cohort). In addition, blood was collected during a single regular visit at the outpatient clinic from 38 stable primary liver transplant recipients 1 to 12 years after transplantation in the Erasmus Medical Centre Rotterdam (The Netherlands) (late post-LTx cohort). Multi-organ transplantation patients were excluded. The occurrence of CMV infection (either primary infection or reactivation) after transplantation was determined either by CMV DNA polymerase chain reaction (PCR) > 50 copies/ml or by CMV IgG seroconversion. All patients gave informed consent, and the study was approved by the Medical Ethics Committee of the Erasmus MC. Nineteen clinically healthy blood bank donors were used as healthy controls and were age-matched with the late post-LTx cohort.

Cell culture

Peripheral blood mononuclear cells (PBMC) from patients and healthy individuals were isolated using Ficoll Hypaque density gradient centrifugation. From the same patients, cryopreserved PBMC collected before LTx (pre-LTx), that were available in our bio bank, were used for baseline measurements. Cryopreserved splenocytes, isolated according to standard procedures (27) from splenic tissue of liver transplant donors, were available in our bio bank as well. CD40-activated B cells were expanded from donor splenocytes, as described previously (27), and used as stimulator cells in allogeneic T-cell stimulation assays. Only B cells containing <1% $CD3^+$ T cells were used. PBMC and expanded B cells were cryopreserved at -135°C until further use.

Flow cytometry

Flow cytometry was performed to determine T-cell subsets and co-inhibitory receptor expression. For analysis of $CD4^+$ and $CD8^+$ T cells, isolated PBMC were stained with anti-CD3-horizonV500 (UCHT1, BD Biosciences, Erembodegem, Belgium), anti-CD4-APC-H7 (SK3, BD Biosciences), and anti-CD8-efluor450 (RPA-T8, eBioscience, Vienna, Austria). To distinguish naive and memory T-cell subsets, cells were stained with anti-CCR7-FITC (150503, R&D systems, Abingdon, United Kingdom) and anti-CD45RO-PerCP-Cy5.5 (UCHL1, Biolegend, London, United Kingdom). Naive T cells (Tn) were defined as CD45RO-$CCR7^+$ T cells; central memory T cells (Tcm) as $CD45RO^+CCR7^+$; effector memory T cells (Tem) as $CD45RO^+$CCR7- and terminally differentiated T cells (Temra) as CD45RO-CCR7- (28). Surface expression of co-inhibitory receptors was determined by staining cells with anti-CD279(PD1)-PECy7 (J105, eBioscience), anti-CD223(LAG3)-PE (polyclonal, R&D systems), anti-CD160-PE (688327, R&D systems) and anti-CD244-APC (eBioDM244, eBioscience). Flow cytometry was performed using a FACS Canto II (BD Biosciences). Gates for PD1 expression were set using an isotype-matched control antibody and gates for LAG3, CD160 and CD244 expression were set on distinct positive populations. For analysis FACS Diva software was used (BD Biosciences).

Allogeneic T-cell stimulation using PBMC

To quantify allo-reactive T-cell responses, PBMC were labeled with 0.5 µM CFSE (Invitrogen, Paisley, United Kingdom) and 1*105 recipient PBMC were stimulated with 2*105 irradiated (30 Gy) donor-derived or third party-derived CD40-B cells. Third party CD40-B cells were expanded from splenocytes of an individual having the same number of HLA mismatches with the patient as the number of mismatches between patient and donor, but completely mismatched with the donor on HLA-A, B, and DR (27). Co-cultures were performed in 96-wells U-bottom plates in a final volume of 200 µl B-cell medium (IMDM + 10% human serum + 1% Penicillin/Streptomycin (Gibco) + 1% Insulin/Transferrin/Selenium (Gibco)) (27). In addition, to determine responses to polyclonal stimulation,

PBMC were stimulated with PHA (5µg/ml, Murex, Paris, France). Each assay was performed in duplicate. Flow cytometric analysis was performed after 5 days of culture at 37°C and 5% CO2. Cells were washed with PBS (Lonza) and staining for cell viability was performed using LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen), according to the manufacturer's protocol. Cells were then stained with anti-CD3-PerCP-Cy5.5 (UCHT1, BD Biosciences), anti-CD4-APC-H7 (SK3, BD Biosciences), anti-CD8-efluor450 (RPA-T8, eBioscience) to distinguish T cells, and anti-CD19-horizonV500 (HIB19, BD Biosciences) to exclude B cells. Cytotoxic degranulation was detected using CD107a-APC (eBioscience), added during the last 15 hours of the co-cultures. Cells were analyzed for proliferation using CFSE-dilution patterns, and for phenotype on a BD FACS Canto II Flow cytometer (BD Biosciences, San Jose, CA). For analysis of phenotypic markers we used FACS Diva software (BD Biosciences). Precursor frequencies (PF), which is the proportion of the cells that respond to the stimulus, of allo-reactive $CD4^+$ and $CD8^+$ T cells were calculated using ModFit LT® software (Verity Software House, USA), as previously described (27). From duplicate assays, average PF were calculated.

Allogeneic T-cell stimulation of sorted T cells

To compare the proliferative capacities of $CD8^+CD244^+$ and $CD8^+CD244$- T cells, post-LTx PBMC of patients of the late cohort were thawed and labeled with 0.5 µM CFSE. CFSE-labeled PBMC were stained with anti-CD3-PerCP-Cy5.5 (UCHT1, BD Biosciences), anti-CD4-APC-H7 (SK3, BD Biosciences), anti-CD8-efluor450 (RPA-T8, eBioscience) and anti-CD244-APC (eBioDM244, eBioscience), and $CD8^+CD244^+$, $CD8^+CD244$-, and $CD4^+$ T cells were purified by flowcytometric sorting, using a FacsAria Cell Sorter (BD Biosciences). Only cells with purity >95% were used. Purified $CD8^+CD244^+$ or $CD8^+CD244$- T cells (2*104) together with purified autologous $CD4^+$ T cells (2*104) were stimulated with 1.6*105 irradiated (30 Gy) donor CD40-B cells or third party CD40-B cells, as described above. To study the role of co-inhibitory receptor-ligand interactions in allogeneic T-cell responses, ligands of co-inhibitory receptors were blocked by addition of neutralizing anti-CD270 (HVEM/TNFRSF14) (Clone 94801, R&D systems (29)), or anti-CD48 (eBio156-4H9, eBioscience (10, 11)) antibodies, either alone or in combination to selected allogeneic T-cells stimulations. After 5 days of culturing, cells were stained and ModFit analyses were performed as described above.

Determination of CMV-specific T cells

To determine frequencies of CMV-specific $CD8^+$ T cells in PBMC and to assess whether these cells expressed CD244, 1*106 PBMC were stained with a mixture of HLA-A*01:01, A*02:01, A*11:01, A*24:02, B*07:02, B*08:01, and B*35:01 MHC class I tetramers loaded with Pp50-derived, Pp65-derived and IE-derived peptides (Department of Hematology, Leiden University Medical Center, The Netherlands), depending on the HLA-types of

the patient. The following peptides were used: Pp50: VTEHDTLLY (HLA-A0101); Pp65: YSEHPTFTSQY (HLA-A0101), NLVPMVATV (HLA-A0201), ATVQGQNLK (HLA-A1101), AYAQKIFKIL (HLA-A2402), RPHERNGFTVL (HLA-B0702), TPRVTGGGAM (HLA-B0702), and IPSINVHHY (HLA-B3501); IE1: QIKVRVDMV (HLA-B0801) and ELRRKMMYM (HLA-B0801). In addition, cells were stained with anti-CD4-PerCP (clone Leu3A SK7, BD Biosciences), anti-CD8-Pacific Blue (clone RPA-T8, BD Biosciences) and anti-CD244-APC (eBioDM244, eBioscience). Flow cytometry was performed using a LSRII (BD Bioscience) and data were analyzed using FACS Diva software.

Statistical analysis

All data are presented as means + SEM. All data sets were tested for normal Gaussian distribution using the Shapiro-Wilk normality test. Significance of differences between paired observations was tested using the paired t-test for normally distributed data or the Wilcoxon signed rank test for non-normally distributed data. Differences between unrelated groups were tested using the Mann-Whitney U test. Statistical analyses were performed using GraphPad Prism (Version 5.01, GraphPad Software Inc, San Diego, CA). Multivariate analysis was performed using linear regression in SPSS for Windows (version 21.0 software package). A p-value <0.05 was considered statistically significant.

RESULTS

Rapid and sustained increase of CD244 and CD160 expression on circulating T cells after LTx

To investigate whether expression of co-inhibitory receptors on T cells changed after LTx, we first analyzed the longitudinal course of the expression of 5 well-known co-inhibitory receptors, namely PD1, LAG3, TIM3, CD160 and CD244 (5), on circulating $CD4^+$ and $CD8^+$ T cells in 19 patients during the first 6 months after LTx. Patient characteristics are depicted in Table I and described in Materials and Methods. Because TIM3 was hardly expressed on T cells in any of the individuals, we excluded TIM3 from further analyses (data not shown). Representative FACS plots are shown in Figure 1A.

Already 1 month after LTx a slight rise in expression of PD1 on $CD4^+$ T cells and CD244 on $CD8^+$ T cells was observed (Figure 1B). At 6 months post-LTx, expression of PD1 on $CD4^+$ T cells had returned to baseline level, while CD244 expression on $CD8^+$ T cells was further increased. In addition, expression of CD244 on $CD4^+$ T cells and of CD160 on $CD8^+$ T cells were significantly increased at 6 months post-LTx. Longitudinal LAG3 expression levels tended to rise at 1 month post-LTx on both $CD4^+$ ($p=0.145$) and $CD8^+$ ($p=0.138$) T cells, but at 6 months post-LTx returned to levels similar to pre-LTx (Figure 1B).

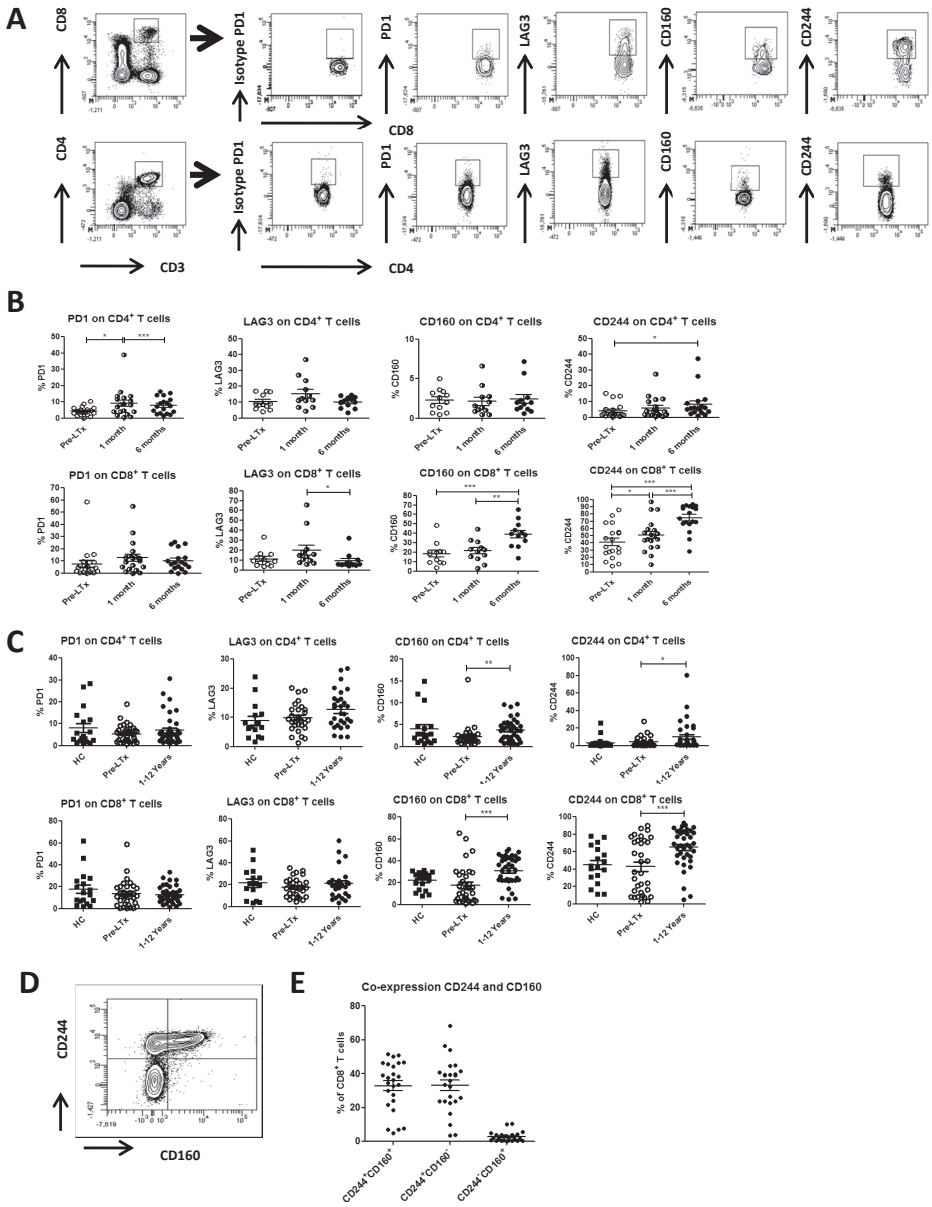

Figure 1.

Expression of co-inhibitory receptors on circulating T cells after LTx. (A) Representative FACS plots showing analysis of co-inhibitory receptors on $CD4^+$ and $CD8^+$ T cells (B) Expression of co-inhibitory receptors on circulating $CD4^+$ and $CD8^+$ T cells of 19 liver transplant recipients before (pre-LTx) and early (1-6 months) post-LTx. (C) Expression of co-inhibitory receptors on circulating $CD4^+$ and $CD8^+$ T cells of healthy controls (HC) and 38 liver transplant recipients before (pre-LTx) and 1-12 years post-LTx. (D) Representative FACS plot showing co-expression of CD244 and CD160 on circulating $CD8^+$ T cells (E) Co-expression of CD244 and CD160 on circulating $CD8^+$ T cells in patients late after LTx (n=25). Each dot represents 1 patient, and lines indicate mean with standard error of the mean. *p<0.05, **p<0.005, ***p<0.0005

To establish whether the changes observed in the first 6 months after LTx were sustained later after LTx, we assessed co-inhibitory receptor expression on circulating T cells in blood samples collected from 38 patients 1 to 12 years after LTx and compared it with expression before LTx. Patient characteristics are depicted in Table I. We found no significant differences in PD1 and LAG3 expression on both $CD4^+$ and $CD8^+$ T cells between pre-LTx and post-LTX samples. However, CD160 and CD244 expression were increased late after LTx on both $CD4^+$ and $CD8^+$ T cells (Figure 1C). In addition, we found that CD160 and CD244 were strongly co-expressed on $CD8^+$ T cells late after LTx: CD160 was mainly expressed on $CD244^+$ $CD8^+$ T cells and almost no $CD160^+CD244$- T cells were observed (Figure 1D-E). Interestingly, expression of co-inhibitory receptors on T cells did not differ between patients pre-LTx and healthy age-matched controls (Figure 1C), indicating that liver disease had no influence on expression levels of co-inhibitory receptors.

Collectively, early after LTx a slight rise in PD1 expression on $CD4^+$ T cells was found, which was not sustained, while the early increase of CD160 and CD244 expression on both $CD4^+$ and $CD8^+$ T cells was sustained late after LTx. We therefore further focused on these two co-inhibitory receptors.

Enhanced expression of CD244 and CD160 on circulating T-cell subsets after LTx

Since CD244 and CD160 expression is low or absent on naive T cells and increases progressively with memory differentiation state of T cells (11, 30), we assessed whether the distribution of circulating naive and memory T-cell subsets changed after LTx (Figure 2A; Materials and Methods). After LTx, a significant reduction of $CD4^+$ and $CD8^+$ Tn was observed, while $CD4^+$ Tem and Temra and CD8+ Temra significantly increased (Supplemental Figure 1). Therefore, to establish whether the upregulation of CD160 and CD244 after LTx was due to the changes in T-cell subset distribution, we determined the expression of these co-inhibitory receptors on each individual T-cell subset.

In the early post-LTx cohort, we observed a significant increase in CD160 expression on $CD8^+$ Tcm and Tem 6 months post-LTx (Figure 2B). In the late post-LTx cohort, CD160 expression significantly increased after LTx on $CD4^+$ Tem and $CD8^+$ Tcm, Tem and Temra (Figure 2C).

On $CD4^+$ Tem and Temra and on all $CD8^+$ T-cell subsets we found increasing expression of CD244 during the first 6 months after LTx in the early post-LTx cohort (Figure 2B). In the late post-LTx cohort, CD244 expression on $CD4^+$ and $CD8^+$ Tem and Temra increased significantly after LTx (Figure 2C).

Taken together, these results show that the observed upregulation of CD160 and CD244 expression on circulating T cells after LTx was not only caused by a shift in T-cell subset distribution, but was also due to increased expression on the individual T-cell subsets.

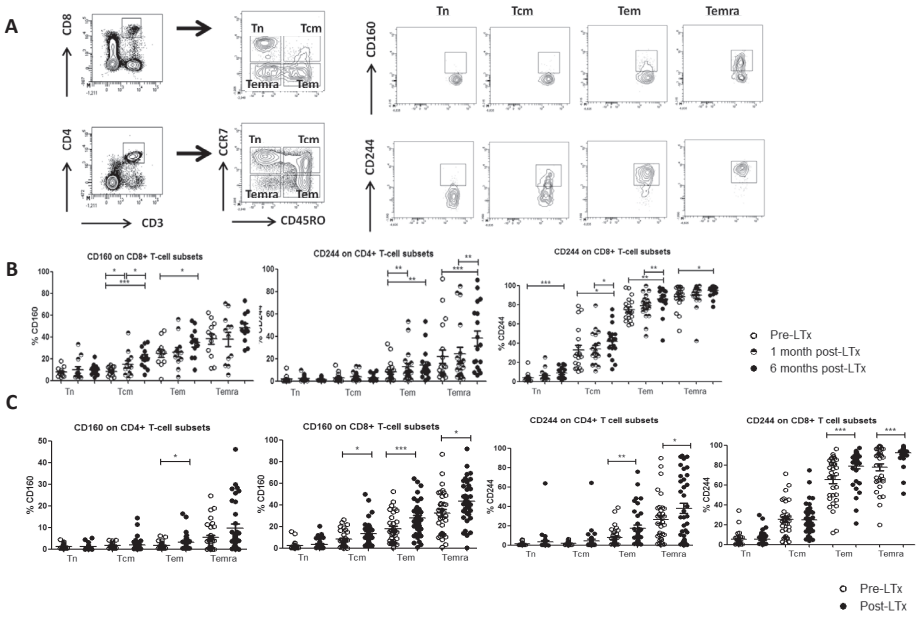

Figure 2.
Expression of co-inhibitory receptors on circulating $CD4^+$ and $CD8^+$ T-cell subsets before and after LTx. (A) Representative FACS plots showing the gating strategy of naive T cells (Tn), central memory T cells (Tcm), effector memory T cells (Tem), and terminally differentiated T cells (Temra) and expression of CD160 and CD244 on circulating $CD8^+$ T-cell subsets. (B) Expression of CD160 and CD244 on different T-cell subsets in patients of the early post-LTx cohort (C) Expression of CD160 and CD244 on different T-cell subsets in patients of the late post-LTx cohort. Each dot represents 1 patient, and lines indicate mean with standard error of the mean. *p<0.05, **p<0.005, ***p<0.0005

Upregulation of CD244 on $CD8^+$ T cells after LTx is associated with CMV infection

Next, we focused on the expression of CD244 and CD160 in patients late after LTx. Since expression of CD244 and CD160 on T cells increases with age (31) and their expression can also be induced by viral infections, such as HBV, HCV and CMV (5, 10, 14, 32-34) we first asked whether the increasing expression of these co-inhibitory receptors after LTx was related to age of the patients, underlying disease (including chronic viral hepatitis), time after LTx, or CMV infection after LTx. Importantly, for this study CMV infection was only regarded as relevant when occurring between LTx and collection of the post-LTx blood sample. Multivariate linear regression analysis showed that the increases in CD160 and CD244 expression (delta expression = expression post-LTx minus expression pre-LTx) were not significantly associated with patient age, time after LTx, or underlying disease. However, upregulation of CD244, but not CD160, on $CD8^+$ T cells after LTx showed a significant positive association with CMV infection after LTx (p=0.004) (Table II). Although we focused on the expression of CD244 and CD160, we also analyzed the association

between CMV infection and the expression of PD1 and LAG3, but no increase in their expression levels was found in CMV infected patients.

Figure 3A shows that the expression of CD244 on CD8$^+$ T cells was significantly upregulated after LTx in patients with CMV infection, but not in patients without CMV infection after LTx. The increases in CD244 expression observed in patients without CMV infection were small (on the average only 7%), while a significantly higher average increase of 38% was observed in patients with CMV infection (Figure 3B). We therefore conclude that CMV infection importantly contributes to the rise in CD244 expression on CD8$^+$ T cells after LTx. Together, our data suggest that strong CD244 expression is induced by CMV-infection early after LTx and that CD244-expression remains high, even many years after CMV infection is cleared. To verify this, we determined CD244 expression levels on CD8$^+$ T cells at 1 year post-LTx in a subgroup of CMV-infected patients (n=6) of the long-term cohort. As shown in Figure 3C, expression of CD244 was already increased in these patients at 1 year after LTx. Although expression levels showed a partial decrease in blood samples taken at 6-11 years post-LTx, they remained significantly higher than pre-LTx expression levels. These data indicate that CMV infection, which occurs predominantly in the first 9 months after transplantation, induces accumulation of CD8$^+$ T cells expressing CD244, and after the clearance of infection (latency) these CD8$^+$CD244$^+$ T cells persist.

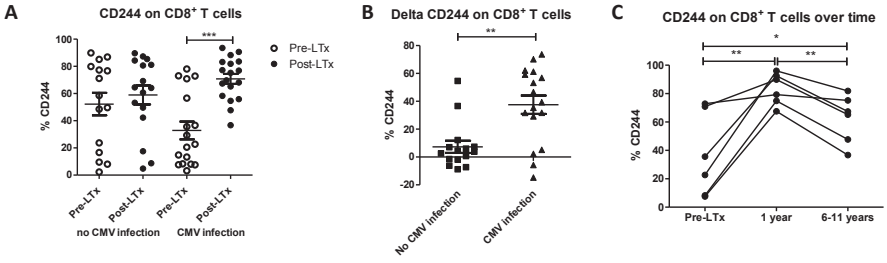

Figure 3.
Expression of CD244 on CD8$^+$ T cells before and after LTx in patients of the late post-LTx cohort with and without CMV infection after LTx. (A) Expression of CD244 on CD8$^+$ T cells before and after LTx in patients of the late post-LTx cohort with and without CMV infection after LTx. (B) Increase of CD244 expression on CD8$^+$ T cells, i.e. delta CD244 (= CD244 expression post-LTx minus pre-LTx expression) in patients with and without CMV-infection after LTx. (C) Expression of CD244 on CD8$^+$ T cells before (pre-LTx), at 1 year, and at 6-11 years post-LTx in a subgroup of patients with CMV infection (n=6) of the long-term cohort. Each dot represents 1 patient, and lines indicate mean with standard error of the mean. *p<0.05, **p<0.005, ***p<0.0005

Reduced allogeneic CD8$^+$ T-cell responses in patients with CMV infection after LTx

Since we found that CMV infection after LTx was associated with upregulation of CD244 expression on CD8$^+$ T cells after LTx, we asked whether CMV infection also affected the allo-reactivity of these cells. We therefore determined post-LTx CD8$^+$ T-cell

allo-responses in patients of the late post-LTx cohort with and without CMV infection, by co-culturing CFSE-labeled patient PBMC with CD40-activated B cells from their liver transplant donors or from an HLA-mismatched third party. After 5 days, proliferation and effector function of $CD8^+$ T cells were assessed. PF of proliferating cells were calculated using Modfit software (27), and representative examples of Modfit proliferation analyses are shown in Figure 4A. As depicted in Figure 4B, PF of $CD8^+$ T cells proliferating in response to donor allo-antigens were significantly lower in PBMC from patients with

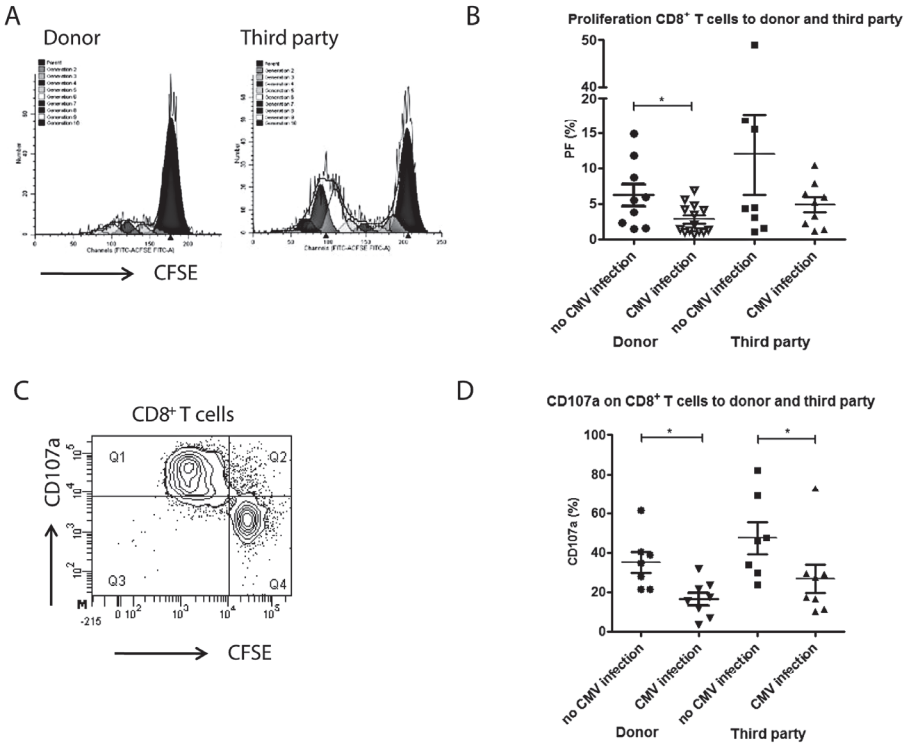

Figure 4.

Allogeneic proliferative and cytotoxic degranulation responses of $CD8^+$ T cells from patients with or without CMV infection after LTx. (A) Representative Modfit analysis plots showing $CD8^+$ T-cell proliferation after 5 days of stimulation with donor-derived or 3rd party-derived CD40-activated B cells. (B) Precursor frequencies (PF) of proliferating $CD8^+$ T cells in post-LTx PBMC of patients with or without CMV infection after LTx in response to donor (n=14 with CMV and n=9 without CMV) or third party (n=9 with CMV and n=8 without CMV) allo-antigens. Blood samples were collected 1-12 years after LTx (median 7 years). (C) Representative FACS plots showing CD107a expression on CFSE-labeled $CD8^+$ T cells after 5 days of stimulation with donor-derived CD40-activated B cells. (D) CD107a expression on both proliferated and non-proliferated $CD8^+$ T cells as percentage of all $CD8^+$ T cells in post-LTx PBMC of patients with (n=8) or without (n=7) CMV infection after LTx in response to donor or third party allo-antigen. Each dot represents 1 patient, and lines indicate mean with standard error of the mean. *$p<0.05$

CMV infection than from patients without CMV infection after LTx, with the same trend (p=0.213) in the responses to third party allo-antigens.

To assess cytotoxic effector function of $CD8^+$ T cells in both patient categories, we determined their cytotoxic degranulation capacity by analyzing CD107a surface expression at the end of the co-cultures (Figure 4C). After stimulation with donor or third party allo-antigens, $CD8^+$ T cells of patients with CMV infection after LTx showed significantly lower levels of CD107a expression than $CD8^+$ T cells of patients without CMV infection (Figure 4D). More specifically, CD107a expression in non-proliferating cells was lowered in patients with CMV-infection, but not CD107a expression in proliferating $CD8^+$ T cells (Supplemental Figure 2). These data demonstrate the existence of circulating $CD8^+$ T cells that do not proliferate but are still capable of cytotoxic degranulation in response to allo-antigens, and show that the decreased allogeneic cytotoxic degranulation capacity in CMV-infected patients was confined to these non-proliferating cells.

Together, these data demonstrate a reduction of allogeneic $CD8^+$ T-cell proliferative and cytotoxic degranulation responses in LTx patients with CMV infection after LTx, and show that CMV-infection induces accumulation of a population of dysfunctional $CD8^+$ T cells which does neither proliferate, nor degranulate in response to allo-stimulation.

$CD244^+$ $CD8^+$ T cells show impaired proliferative responses to allogeneic stimulation

Since we found that CMV infection was associated with a strong rise in CD244 expression on circulating $CD8^+$ T cells and with hyporesponsiveness of $CD8^+$ T cells to allo-antigens after LTx, we wondered whether CD244 expression hallmarks a subpopulation of $CD8^+$ T cells with reduced functionality. To test this hypothesis, CFSE-labeled CD244- and $CD244^+$ $CD8^+$ T cells, as well as $CD4^+$ T cells, were sorted from 17 LTx patients of the late post-LTx cohort. The sorted $CD8^+$ T-cell subsets were co-cultured with autologous $CD4^+$ T cells to provide CD4-help to the $CD8^+$ T cells, and stimulated with allogeneic CD40-activated B cells either derived from the donor or from an HLA-mismatched third party. In addition, both sorted subsets were stimulated with PHA. After 5 days of culture, cells were harvested and proliferation was measured. Significantly lower numbers of $CD244^+$ T cells than CD244- T cells proliferated in response to PHA (Figure 5A; p=0.0001). Similarly, significantly less $CD244^+$ T cells than CD244- T cells proliferated in response to allogeneic stimulations (p=0.002 for donor and p=0.023 for third party stimulation). The impaired proliferative responses of $CD244^+$ T cells were independent of the allo-antigenic source, as differences between $CD244^+$ and CD244- T cells were similar in response to donor and third party stimulations (Figure 5B). To assess whether blocking the interaction of CD244 with its ligand CD48 could restore the proliferative capacity of $CD244^+$ $CD8^+$ T cells, we repeated the above described experiments in a subgroup of patients in the presence of blocking antibodies directed against CD48. In addition, we studied the effect of blocking

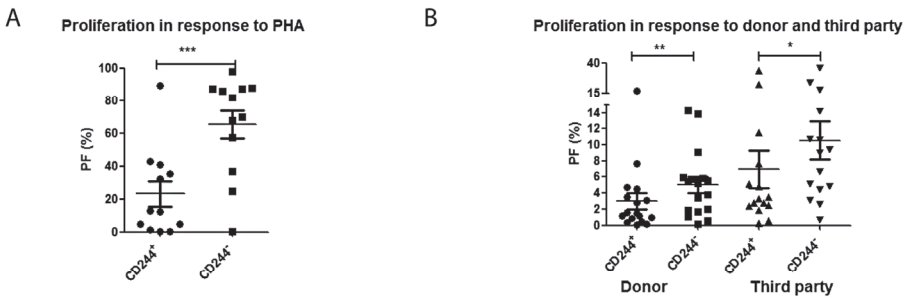

Figure 5.
Proliferation of CD244$^+$ versus CD244- CD8$^+$ T cells in response to polyclonal and allogeneic stimulation. (A) Precursor frequencies (PF) of sorted CD244$^+$ versus CD244- CD8$^+$ T cells in response to polyclonal stimulation (PHA). (B) Precursor frequencies of CD244$^+$ versus CD244- CD8$^+$ T cells of patients post-LTx in response to donor and third party stimulation. Cells were sorted from PBMC collected from 17 patients 2-10 years after LTx (median 6.2 years). Each dot represents 1 patient, and lines indicate mean with standard error of the mean. *p<0.05, **p<0.005, ***p<0.0005

the interaction of CD160, which is co-expressed with CD244, with its ligand HVEM during culture. Both CD48 and HVEM were expressed on CD40-activated B cells (data not shown). After 5 days, we did not find any difference in allogeneic proliferation of both CD244$^+$ and CD244- CD8$^+$ T cells between conditions with and without blocking antibodies (data not shown). Collectively, these data suggest that CD244 expression marks a subset of dysfunctional CD8$^+$ T cells, but the receptor itself and the co-expressed CD160 receptor do not mediate the dysfunctionality.

CD244$^+$ CD8$^+$ T cells contain the majority of CMV-specific cells

Since upregulation of CD244 on circulating CD8$^+$ T cells was strongly associated with CMV infection after LTx and CD8$^+$CD244$^+$ T cells were dysfunctional, we analyzed whether CD244$^+$ CD8$^+$ T cells contained CMV-specific cells. We therefore co-stained PBMC from 5 CMV-experienced LTx patients from our study with MHC class I tetramers loaded with CMV-peptides and CD244 mAb, as described in Materials and Methods. A representative FACS plot is shown in Figure 6A. We found that the CD244$^+$ CD8$^+$ T-cell population contained the majority of CMV-tetramer positive cells; significantly more than the CD244- CD8$^+$ T-cell population (Figure 6B, p=0.033). These data support a causal relationship between CMV infection and the expansion of dysfunctional CD8$^+$ CD244$^+$ T cells after LTx.

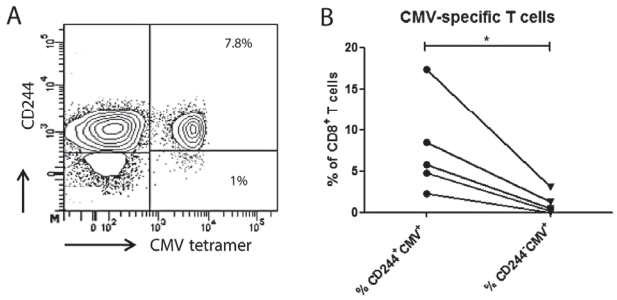

Figure 6.
CMV-specific cells within CD244$^+$ and CD244- CD8$^+$ T-cell populations. (A) Representative FACS plots showing CD244$^+$ and CD244- CMV-tetramer positive cells CD8$^+$ T cells. (B) Percentage CD244$^+$ and CD244- CMV-tetramer positive cells of CD8$^+$ T cells of CMV-positive patients post-LTx (n=5).

DISCUSSION

In this study, we showed that the co-inhibitory receptor CD160 was upregulated on circulating memory CD8$^+$ T cells, while the co-inhibitory receptor CD244 was upregulated on both CD4$^+$ and CD8$^+$ memory T cells of patients early after LTx. In addition, we found that the increased expression of both receptors was sustained late after LTx. In contrast, PD1 was transiently upregulated on CD4$^+$ T cells 1 month after LTx, but its expression normalized already at 6 months after LTx. Our original hypothesis postulated that long-term persistence of a high load of allo-antigens after LTx may induce exhaustion of allogeneic T-cells. In contrast to this hypothesis, impaired allogeneic CD8$^+$ T-cell responses and strong upregulation of CD244 on CD8$^+$ T cells after LTx were restricted to patients with CMV infection after LTx. Expression of CD244 on the majority of the CMV-specific CD8$^+$ cells in patients with CMV infection after LTx suggested a causal relationship between CMV infection and the observed expansion of CD8$^+$CD244$^+$ T cells. The observed lower proliferative capacity of CD244$^+$ CD8$^+$ T cells than CD244-CD8$^+$ T cells in response to allogeneic stimulation suggested that the allogeneic CD8$^+$ T-cell hyporesponsiveness in LTx patients after CMV infection is caused by the accumulated CD8$^+$CD244$^+$ T cells. Together, these data suggest that CMV infection after LTx induces persistent accumulation of CD8$^+$CD244$^+$ T cells in the circulation, which display features of senescence or exhaustion, resulting in impaired peripheral CD8$^+$ T-cell responses to allo-antigens in these patients.

The observed association between accumulation of CD8$^+$CD244$^+$ T cells and CMV infection (11, 14, 30, 35) as well as the selective expression of CD160 and CD244 on memory T-cell subsets is consistent with previous studies (4, 11, 30, 36). However, to our knowledge, this is the first study showing that CMV infection after organ transplantation induces

sustained CD244 expression on memory $CD8^+$ T cells and that the resulting $CD8^+CD244^+$ T-cell subset is hyporesponsive to allo-antigens. It has been well-documented that CMV infection induces vast expansion in the circulation of a population of CMV-specific $CD8^+$ T cells which are actively cycling. After establishment of CMV-latency the majority of CMV-specific $CD8^+$ T cells become long-lived terminally differentiated resting T cells with poor proliferative capacity (37, 38). Therefore, we hypothesize that expansion of these cells after LTx occurs during active CMV infection, while they differentiate into long-lived CD244-expressing terminally differentiated T cells with poor proliferative capacity after establishment of CMV-latency. Expression of CD244 is also induced on $CD8^+$ T cells by HIV and HCV infection, and results in impaired $CD8^+$ T-cell responses to viral antigens. However, the impairment of anti-viral $CD8^+$ T-cell responses in these patients can be abrogated by blocking the interaction between CD244 and its ligand CD48 (10, 11, 14). In contrast, our data suggest that the observed allogeneic hyporesponsiveness of CMV-induced $CD244^+CD8^+$ T cells was not mediated by CD244-CD48 interaction, neither by interaction of the co-expressed inhibitory CD160 receptor with its ligand HVEM, since blocking CD48 or HVEM did not lead to abrogation of hyporesponsiveness of $CD8^+CD244^+$ T cells to allo-antigens in experiments with sorted $CD244^+$ T cells. The impaired response of this subset to allo-antigens may be related to its high content of CMV-specific T cells, resulting in lower proportions of T cells with other specificities, including allo-reactive T cells (39-41). In addition, the limited TCR repertoire of CMV-specific cells (39, 42, 43) accumulated in this subset may result in poor cross-reactivity to directly presented allo-antigens. However, these phenomena do not explain the impaired proliferation of the expanded $CD8^+CD244^+$ T cells to PHA (this study), or to CD3/CD28 stimulation in a previous study (30). Interestingly, CMV-induced expansion of $CD8^+$ effector memory cells correlates with a decrease in T-cell telomere length, indicating T-cell senescence (44), and T-cell senescence has particularly been related to impaired proliferative capacity (45). We therefore propose that the observed rise in CD244 expression in LTx patients with CMV infection marks expansion of a subset of highly differentiated but dysfunctional CD8+ T cells, which shows features of senescence or exhaustion. However, its proliferative capacity is hampered by an as yet unknown mechanism.

The observed reduction in allo-reactive $CD8^+$ T-cell responses in LTx patients after CMV infection challenges the broadly accepted notion, based on experimental animal studies, that viral infections stimulate heterologous immunity resulting in increased frequencies of allo-reactive T-cells (46). Indeed, reactivation of CMV infection as well as primary CMV infection abrogate transplant acceptance in mice and rat (47, 48). However, several previously published observations in humans support our finding. First, CMV infection leads to T-cell senescence, and thereby impairs T-cell responses to other antigens and to vaccinations (34, 40, 49-51). Interestingly, CMV infection after LTx is associated with an increased predisposition to develop opportunistic infections (52). Secondly, immune

senescence has been associated with improved kidney allograft survival (53). Thirdly, the majority of $CD8^+CD244^+$ T cells in our patients belong to the Temra subset, and accumulation of circulating $CD8^+$ Temra has recently been shown to be associated with lower risk of acute rejection after kidney transplantation (41). It was not feasible to investigate whether CMV infection or accumulation of $CD8^+CD244^+$ cells were associated with differences in clinical outcome such as graft or patient survival or acute rejection in our long-term study cohort, since all patients have stable graft function and are still alive. In addition, only 5 patients suffered from acute rejection. A larger prospective study is required to investigate associations between CMV infection or rise in CD244 and clinical outcomes.

The mechanism by which CMV infection induces expansion of $CD244^+CD8^+$ memory T cells is as yet unknown, but may be related to bystander effects of inflammatory responses caused by CMV or by its immune evasion strategies. A recent mouse study showed that CD244 is more highly upregulated during secondary than during primary $CD8^+$ T-cell responses, suggesting that T-cell reactivation is required for induction of high CD244 expression (54). In addition, it has been shown that chronic lymphocytic choriomeningitis virus (LCMV) or Toxoplasm infections in mice impair memory T-cell responses against unrelated antigens due to generation of $CD8^+$ Temra. This was caused by increased IFN-type signalling due to chronic inflammation caused by the persistent infections (55). A similar mechanism may be driven by CMV infection after LTx, which also causes inflammation in the graft and in other organs (52), while CMV is able to induce IFN-α production (56). A possible relation between inflammation and reduced allo-responses after LTx is supported by a recent study that showed that chronic HCV patients who are operationally tolerant after LTx over-express type I IFN and Interferon-Stimulated Genes in the liver graft (57). A second explanation for the association between CMV infection and $CD8^+$ T-cell hyporesponsiveness may be that CMV produces viral IL-10 (58), which inhibits expansion of allo-reactive $CD8^+$ T cells. A third explanation may be that the immunological space of the recipient is occupied by large quantities of CMV-specific $CD8^+$ Temra that compete with and thus hamper the expansion of T cells with other specificities (59). However, these explanations remain speculative, and further research is needed to decipher the mechanisms by which CMV infection induces expansion of $CD8^+CD244^+$ memory T cells, which is beyond the scope of the present study.

In contrast to the sustained increase in CD244 and CD160 expression on circulating T cells after LTx, we found that PD1 was only transiently upregulated following LTx. As PD1 can be upregulated by TCR-activation, this finding may be explained by the early and transient activation of donor-specific T cells after LTx that we observed previously (27). However, the use of calcineurin inhibitors (CNI) by the majority of our patients may prevent sustained upregulation of PD1, since PD1 induction by TCR-ligation involves NFAT-signaling, which is inhibited by CNI (32). PD1 upregulation is also prevented by

mTOR inhibitors (60), immunosuppressive drugs used by a small group of patients in our cohorts. LAG3 and TIM3 expression did not show an increase after LTx, but we do not know whether this is related to the use of immunosuppressive drugs since no evidence exists on the effect of immunosuppressive drugs on the expression of these receptors.

A limitation of our study is that we were not able to link the $CD8^+$ T-cell hyporesponsiveness to an immunologically tolerant state towards the liver allograft. To investigate the clinical impact of the findings presented in our study, it will be interesting to determine the implications of CMV infection in LTx patients on the success rate of withdrawal of immunosuppressive drugs. A prospective study in which immunosuppressive drugs are weaned off is needed to investigate this.

In conclusion, in this study we showed that CMV infection after LTx was associated with the expansion of $CD8^+CD244^+$ T-cells with impaired proliferative capacity in response to allo-antigen, causing allogeneic $CD8^+$ T-cell hyporesponsiveness. These results suggest that CMV infection may hamper T-cell immunity and thereby promote immunological graft acceptance after LTx.

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Table I. Demographic and clinical characteristics of patients included in the early post-LTx and late post-LTx cohorts

Early post-LTx cohort	Total: 19 patients
Recipient age (median, range), years	43 (25-63)
Recipient gender, female (no, %)	10 (53)
Underlying disease (no, %)	
AHF	4 (21)
HCC	0 (0)
PBC/PSC/AIH/SBC	6 (32)
HBV/HCV	4 (21)
Alcoholic cirrhosis	2 (10)
Others	3 (16)
Donor age (median, range), years	44 (15-77)
Donor gender, female (no, %)	10 (53)
Basiliximab as induction immunosuppression (no, %)	10 (53)
Immunosuppressive treatment (no, %, trough level at 6 months (median, range) µg/l)	
Cyclosporin A	7 (37) 175 (25-300)

Table I. Demographic and clinical characteristics of patients included in the early post-LTx and late post-LTx cohorts (continued)

Early post-LTx cohort	**Total: 19 patients**
Tacrolimus	11 (58) 9.1 (4.5-25)
Everolimus	2* (10) 11.7 (6.6-16.8)
Mycophenolate mofetil	1‡ (5) 2.96
No immunosuppression	0 (0)
Late post-LTx cohort	**Total: 38 patients**
Recipient age (median, range), years	46 (20-64)
Recipient gender, female (no, %)	16 (42)
Underlying disease (no, %)	
AHF	1 (3)
HCC	1 (3)
PBC/PSC/AIH	17 (45)
HBV/HCV	1 (3)
Alcoholic cirrhosis	8 (21)
Others	10 (26)
Donor age (median, range), years	43 (12-77)
Time after LTx (median, range), years	7 (1-12)
Donor gender, female (no, %)	18 (47)
Basiliximab as induction immunosuppression (no, %)	27 (71)
Immunosuppressive treatment (no, %, trough level at time of post-LTx blood collection (median, range) µg/l)	
Cyclosporin A	2 (5) levels unknown
Tacrolimus	28 (4) 4.4 (0-9.8)
Everolimus	5† (13) 12.6 (10-15.7)
Rapamycin	1§ (3) 1.14
Mycophenolate mofetil	4‡ (11) levels unknown
No immunosuppression	3 (8)
CMV infection/no infection between LTx and collection of post-LTx blood sample (no, %)	
Infection	20 (53)
No infection	18 (47)

AHF Acute Hepatic Failure; HCC Hepatocellular carcinoma; PBC Primary Biliary Cirrhosis; PSC Primary Sclerosing Cholangitis; AIH Auto-immune hepatitis; SBC Secondary Biliary Cirrhosis; HBV Hepatitis B virus; HCV Hepatitis C virus.

* one patient in the early post-LTx cohort received combination of tacrolimus and everolimus.

‡ one patient in the early post-LTx cohort received combination of tacrolimus and mycophenolate mofetil; 4 patients in the late post-LTx cohort received mycophenolate mofetil of which 2 in combination with tacrolimus.

† 5 patients in the late post-LTx cohort received everolimus of which 2 in combination with tacrolimus.

§ 1 patient in the late post-LTx cohort received rapamycin in combination with tacrolimus.

Table II. Associations of independent covariates with increasing (delta) CD160 and CD244 expression on $CD4^+$ and $CD8^+$T cells in multivariate linear regression analysis

Variable	Beta	p-value
Delta* CD160 on $CD4^+$ T cells		
Age recipient	0.336	0.199
Time after LTx (years)	0.171	0.492
Underlying disease	0.047	0.842
CMV infection after LTx	0.357	0.165
Delta CD160 on $CD8^+$ T cells		
Age recipient	0.239	0.368
Time after LTx (years)	-0.287	0.269
Underlying disease	-0.048	0.843
CMV infection after LTx	0.434	0.106
Delta CD244 on $CD4^+$ T cells		
Age recipient	0.469	0.069
Time after LTx (years)	0.180	0.450
Underlying disease	-0.066	0.769
CMV infection after LTx	0.232	0.337
Delta CD244 on $CD8^+$ T cells		
Age recipient	-0.223	0.251
Time after LTx (years)	0.161	0.388
Underlying disease	-0.147	0.410
CMV infection after LTx	0.631	**0.004**

*Delta expression = expression post-LTx minus expression pre-LTx.

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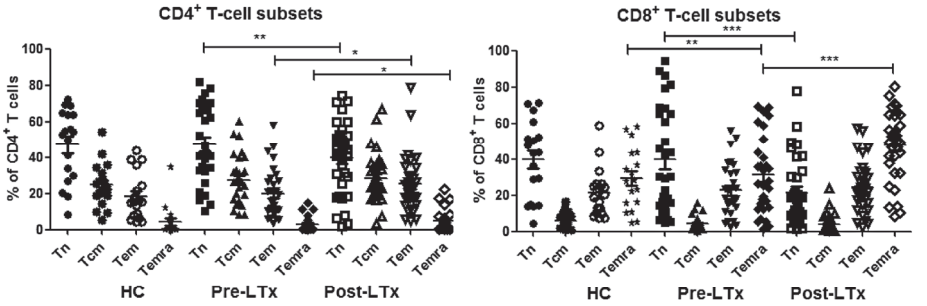
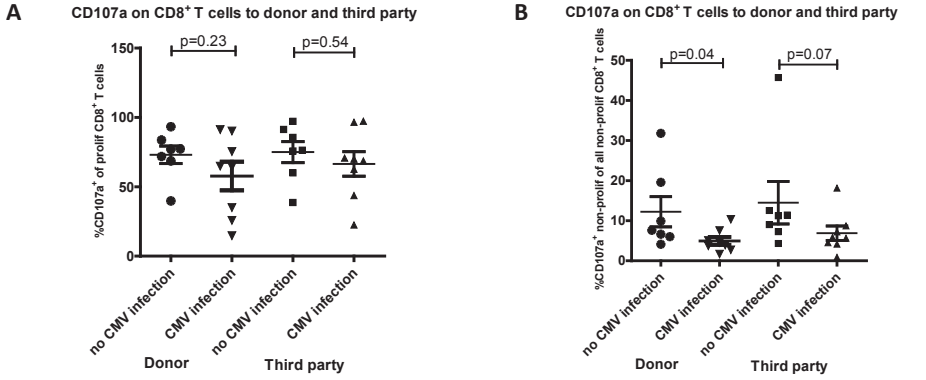


Supplemental Figure 1.
Subset distribution of circulating $CD4^+$ and $CD8^+$ T cells of healthy controls and patients before and late after LTx. Differentiation stages of T cells in LTx patients of the late post-LTx cohort (n=38) before and after LTx, and in healthy control subjects (HC) (n=18).



Supplemental Figure 2.
Allogeneic cytotoxic degranulation responses of CD8+ T cells from patients with or without CMV infection after LTx (A) CD107a expression on proliferated $CD8^+$ T cells as percentage of all proliferated $CD8^+$ T cells in post-LTx PBMC of patients with (n=8) or without (n=7) CMV infection after LTx in response to donor or third party allo-antigen. (B) CD107a expression on non-proliferated $CD8^+$ T cells as percentage of all non-proliferated $CD8^+$ T cells in post-LTx PBMC of patients with (n=8) or without (n=7) CMV infection after LTx in response to donor or third party allo-antigen. Each dot represents 1 patient, and lines indicate mean with standard error of the mean.

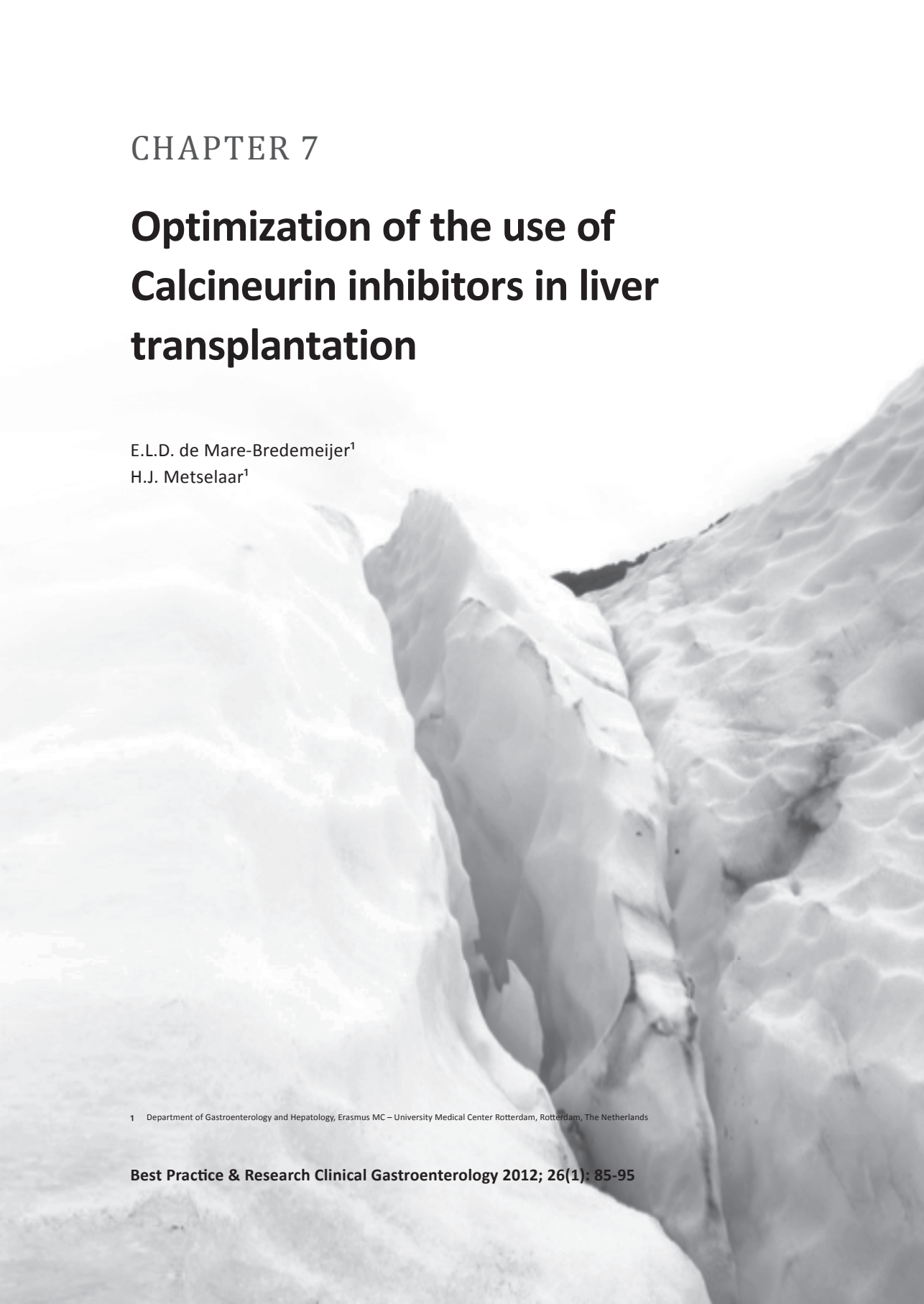
PART III

Optimization of immunosuppression after liver transplantation

CHAPTER 7

Optimization of the use of Calcineurin inhibitors in liver transplantation

E.L.D. de Mare-Bredemeijer[1]
H.J. Metselaar[1]



1 Department of Gastroenterology and Hepatology, Erasmus MC – University Medical Center Rotterdam, Rotterdam, The Netherlands

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ABSTRACT

Calcineurin inhibitors (CNIs), such as cyclosporin A and tacrolimus, are the cornerstone of maintenance immunosuppressive regimens in liver transplantation. CNIs prevent rejection by inhibition of calcineurin, via which lymphocyte proliferation and interleukin (IL)-2 production is prevented. Tacrolimus is now the first-choice immunosuppressant after liver transplantation, since it is associated with fewer episodes of rejection than cyclosporin A. In this review we will discuss interindividual differences, which influence tacrolimus metabolism. Because of these factors and the narrow therapeutic index of tacrolimus, monitoring of drug trough levels is necessary. Furthermore, we will discuss studies concerning conversion from the tacrolimus twice daily to tacrolimus once daily formulation in stable LT patients. Due to adverse effects of CNIs, such as chronic renal failure, hypertension, de novo malignancy and new-onset diabetes mellitus, CNI minimization strategies have been developed, which will be discussed too.

Calcineurin inhibitors – history

Calcineurin inhibitors (CNIs), since their introduction in the 1980s, have been the cornerstone of maintenance immunosuppressive regimens in liver transplantation. The use of CNIs has substantially decreased the risk of acute rejection and improved short-term outcomes. Cyclosporin A (CsA) was introduced in 1983 as a therapeutic agent for renal allograft rejection andrevolutionized the field of clinical transplantation due to the improved organ transplant survival rate (1). Since that time, its use has expanded to cardiac, liver, heart-lung, and multiple organ transplants as well as to the treatment of certain autoimmune diseases such as psoriasis and insulin-dependent type I diabetes. Use of CsA is limited by its side-effect profile, particularly its chronic nephrotoxicity, and so safer immunosuppressive agents have been investigated. Tacrolimus (FK-506, TAC), a compound of about 100-fold more potency than CsA, was developed and tested in the late eighties for the prevention of liver transplant rejection (2) and (3). In 1994, the US Multicenter FK506 Liver Study Group published a paper comparing CsA and TAC for immunosuppression after liver transplantation (4). The study was important for the development of immunosuppression after liver transplantation. First, the introduction stated that rejection remained an important cause of graft loss and death. Second, the paper reported that graft and patient survival with CsA and TAC was similar, but that TAC was associated with fewer episodes of rejection. Third, it reported that TAC was associated with less adverse events, such as nephrotoxicity and neurotoxicity. From the mid-1990s in more and more centers tacrolimus became the first-choice immunosuppressant after liver transplantation. In 2006, a systematic review of randomized clinical trials (RCT) evaluated the beneficial and harmful effects of immunosuppression with cyclosporin versus tacrolimus for liver transplanted patients (5). This reported that mortality and graft loss at one year were significantly reduced in tacrolimus-treated recipients (Death: RR 0.85, 95% CI 0.73–0.99; graft loss: RR 0.73, 95% CI 0.61–0.86). Tacrolimus reduced the number of recipients with acute rejection (RR 0.81, 95% CI 0.75–0.88) and steroid-resistant rejection (RR 0.54, 95% CI 0.47–0.74) in the first year. Lymphoproliferative disorder or dialysis rates were not different between tacrolimus- and cyclosporin-treated patients. More de novo diabetes (RR 1.38, 95% CI 1.01–1.86) occurred with tacrolimus. The current most used combination is tacrolimus, prednisolone andmycophenolate mofetil with in some centers an induction therapy with IL-2 antagonists basiliximab or dacluzimab. With the use of these immunosuppressants, the incidence of biopsy-proven acute rejection within the first year after transplantation ranges between 19 and 30% (6,7).

Calcineurin inhibitors – mechanisms of action

For understanding the mechanism via which CNIs prevent rejection, it is necessary to understand the mechanism of allograft rejection. T cell recognition of alloantigen is the primary and central event that leads to the cascade of events that result in rejection of

a transplanted organ. Individual T cells (or colonies of identical T cell clones) are monospecific as they recognize only a single peptide antigen presented in the context of Major Histocompatibility Complex (MHC). MHC molecules are peptide complexes, expressed on the surface of a variety of immune cells. T cells recognize portions of protein antigens that have been fragmented into peptides bound to MHC molecules. There are at least two distinct, but not necessarily mutually exclusive, pathways of allorecognition, the direct and indirect pathways. Each leads to the generation of different sets of allospecific T cell clones. In the direct pathway, host T cells recognize intact allo-MHC molecules on the surface of the donor antigen presenting cells. In the indirect pathway, T cells recognize processed alloantigen presented as peptides by host antigen presenting cells (8). After allorecognition by T cells, T cells become activated. Following T cell activation, a number of biochemical events occur within the cytoplasm of the T cell. These pathways ultimately lead to a marked and sustained elevation in intracellular calcium. This elevation in calcium promotes the formation of calcium-calmodulin complexes that activate a number of kinases including the phosphatase calcineurin. Calcineurin dephosphorylates cytoplasmic NFAT (nuclear factor of activated T cells), permitting its translocation to the nucleus, where it binds to the interleukin (IL)-2 promoter sequence and then stimulates transcription of IL-2 mRNA. IL-2 is a Proinflammatory cytokine (9,10). Calcineurin is the ultimate target of both cyclosporin and tacrolimus (11). Cyclosporin is a lipophilic cyclic peptide of 11 amino acids while tacrolimus is a macrolide antibiotic. Both drugs have been isolated from fungi and possess similar suppressive effects on cell mediated and humoural immune responses. CNIs bind with high affinity to a family of cytoplasmic proteins present in most cells: cyclophilins for cyclosporin; and FK binding proteins for tacrolimus (because tacrolimus was initially called FK506). The drug-receptor complex specifically and competitively binds to and inhibits calcineurin. This process inhibits the translocation of a family of transcription factors, leading to reduced transcriptional activation of early cytokine genes for IL-2, tumor necrosis factor alpha (TNF- Alpha), IL-3, IL-4, CD40L, granulocyte-macrophage colony-stimulating factor, and interferon-gamma (12). Ultimately, proliferation of lymphocytes and production of the proinflammatory cytokine IL-2 are reduced. Due to the inhibition of these proinflammatory actions, rejection is prevented.

Tacrolimus metabolism and individual dosing

The therapeutic use of tacrolimus is complicated by its variable oral pharmacokinetic profile. The interindividual variability and a narrow therapeutic index, makes therapeutic drug monitoring of tacrolimus levels very important (13). Tacrolimus is metabolized by cytochrome P450 3A (CYP3A) enzymes in the liver and small intestine – in particular, CYP3A4 and CYP3A5 – and are transported out of cells via P-glycoprotein (ABCB1) (14–16). The main reactions tacrolimus undergoes during metabolism are demethylation

and/or hydroxylation (17). Several individual factors may influence the metabolism. These factors will be discussed here. Gonschior et al studied the influence of kidney and liver function on tacrolimus metabolite patterns in blood in kidney and liver transplant recipients (18). Gonschior et al showed that tacrolimus metabolite concentrations and the metabolite patterns depend on the type of graft (kidney vs liver), time after transplantation and cholestasis. The doses required to obtain tacrolimus blood trough concentrations in the therapeutic range were significantly higher in kidney than in liver graft patients. In liver transplant patients, all detected metabolites were higher during the late postoperative period ('day > 14') compared to earlier. More than 90% of an absorbed tacrolimus dose is eliminated in bile as metabolites. Gonschior et al found that cholestasis leaded to increased trough blood concentrations of tacrolimus metabolites as well as to an alteration of the metabolite pattern, which is caused by an overproportional increase of metabolites such as dideinethyl and didemethylhydroxy tacrolimus. This result indicates that the formation of tacrolimus during cholestasiswas impaired to a lesser extent than excretion of the metabolites into the bile duct. When passage through the biliary membrane is hampered, the metabolites usually excreted into bile accumulate in the hepatocyte and enter the blood. Another factor influencing the metabolism of tacrolimus may be interindividual differences in CYP3A enzymes. Several single nucleotide polymorphisms (SNPs) have been identified in the genes encoding for CYP3A4, CYP3A5 and P-glycoprotein. Based on literature review, Staatz et al (19) stated that variability in CYP3A4 expression due to environmental factors is likely to be more important than patient genotype. Furthermore, The CYP3A5 6986A > G SNP has a well established influence on the pharmacokinetics of tacrolimus. Several studies in kidney, heart and liver transplant recipients have reported an approximate halving of tacrolimus dose-adjusted trough concentrations and doubling of tacrolimus dose requirements in heterozygous or homozygous carriers of aCYP3A5*1 wild-type allele compared to homozygous carriers of a CYP3A5*3 variant allele. Influence of ABCB1 SNPs on the pharmacokinetics of cyclosporin and tacrolimus remains uncertain, with inconsistent results. The majority of studies have only evaluated the effects of individual SNPs; however, multiple polymorphisms may interact to produce a combined effect. Differences in ethnic background may also influence the pharmacokinetics of tacrolimus. Mancinelli et al (20) investigated pharmacokinetics of tacrolimus in African Americans, white and Latin Americans. No significant differences among these groups were found after intravenous administration of the drugs. However, after oral administration the tacrolimus maximum concentration was significantly lower in the African Americans than in the other groups. This finding confirmed what has been shown in other studies; although in these studies the investigated drug was cyclosporin and not tacrolimus (21–26). It is unclear whether these differences are due to differences in CYP3A enzymes or that other factors contribute to the different pharmacokinetics among different ethnic groups. To summarize,

in individual tacrolimus dosing, it is important to take into account that interindividual differences, such as time after liver transplantation, cholestasis, genetic differences in CYP3A enzymes and ethnic background could influence the tacrolimus metabolism. All these factors make therapeutic drug monitoring of tacrolimus levels necessary.

CNIs and hepatitis C (HCV)

The use of CNIs in HCV-positive patients deserves special attention. A major problem in HCVpositive patients undergoing liver transplantation is re-infection of the graft, with HCV. The current standard therapy of HCV, pegylated interferon-α (IFN- α) in combination with ribavirin, has achieved substantial success in primary HCV patients, with half of the patients overall developing a sustained virological response (27). Nevertheless, treatment of HCV recurrence after liver transplantation is much less effective, with approximate sustained virological response rates of only 20% (28,29). Recent studies showed that cyclosporin, but not tacrolimus, could inhibit HCV replication in vitro (30,31). Moreover, several in vitro studies have shown that cotreatment with CsA and IFN-α results in greater, synergistic inhibition of HCV replication (32–34). Furthermore, other studies have suggested that tacrolimus interferes with the antiviral activity of IFN-α in vitro (35,36). Pan et al more extensively investigated the effects of calcineurin inhibitors on IFN-α signalling and antiviral activity in subgenomic and infectious HCV models (37). They found no evidence that either CsA or tacrolimus interferes with IFN-α–induced gene expression or IFN-α–mediated antiviral activity against HCV. Their study confirmed results from earlier studies (30–34) showing that cyclosporin has antiviral activity alone and in combination with IFN-α treatment. Furthermore, treatment with tacrolimus alone showed limited antiviral activity, as shown earlier (30,33,38). Pan et al did not observe any inhibitory effect of tacrolimus on IFN-α signalling or its antiviral activity, in contrast to previous reports (35,36). In conclusion, cyclosporin has antiviral activity alone and in combination with IFN-α treatment. Treatment with tacrolimus has limited antiviral activity, but –according to Pan et al– does not interfere with IFN-α treatment against HCV. In clinical practice, tacrolimus still remains the first-choice immunosuppressant in liver transplant recipients, also in HCV-infected patients.

CNIs and interactions with concomitant medication

As described above, CNIs are metabolized by cytochrome P450 3A enzymes. Because some other drugs affect or are metabolized by these enzymes, important interactions between CNIs and these drugs can occur.We will not discuss all these specific drugs separately, but it is important to know that interactions can occur. This indicates that higher or lower tacrolimus levels may be the result. In clinical practice, always consider possible interactions to prevent unwanted effects.

Tacrolimus twice daily versus once daily

In clinical practice, tacrolimus (Prograf®; Astellas Pharma Europe, Staines, UK) is usually administered twice daily (BID). Extended-release or QD tacrolimus (Advagraf®; Astellas Pharma Europe, Staines, UK) is a prolonged-release formulation that was developed to enable a QD dosing alternative and to have similar safety and efficacy profiles to tacrolimus BID with the potential to improve patient adherence and quality of life. Beckebaum et al studied the adherence in 125 stable LT-recipients who have been converted from a twice daily tacrolimus to a once daily prolonged-release formulation (39). They used a four-item validated questionnaire and a Visual Analog Scale. Patient's preference with the treatment regimen was also assessed by a self-report at the end of the observational period (one year after conversion). Overall nonadherence, reported on at least one of the four queried items decreased from 66.4% at baseline (conversion) to 30.9% one year post conversion ($p < 0.0001$). Timing nonadherence (taking the dose with a delay of >2 h) decreased significantly from baseline (63.6%) to one year (27.3%), $p < 0.0001$. Taking nonadherence decreased from 20% to 8.2% ($p < 0.005$). Drug holidays and dose reduction were not reported frequently and differed not significantly from baseline to one year reports. Furthermore, of the 110 patients who maintained on tacrolimus QD medication throughout the study, 94 reported one or more advantages to conversion to the new formulation. No patients reported preference of reconversion to tacrolimus BID formulation at study completion. Tacrolimus has a narrow therapeutic index (40,41) and its oral bioavailability is highly variable between individuals (42). Systemic exposure to tacrolimus (area under the curve, AUC) is a significant efficacy variable and, therefore, therapy is optimized on an individual patient basis by monitoring trough levels as surrogate markers of exposure. Tacrolimus pharmacokinetics (PK) have been compared between tacrolimus QD and tacrolimus BID in de novo and stable kidney, liver and heart patients (43–45). Mean $AUC_{0\text{-}24}$ of tacrolimus on day 1 after transplantation was approximately 30% lower for tacrolimus QD than tacrolimus BID, but was comparable by day 4. There was a good correlation and a similar relationship between $AUC_{0\text{-}24}$ and C_{min} for both formulations. Efficacy and safety data were also comparable. Recently, Fischer et al performed a randomized, phase 2, multicenter prospective trial in primary liver transplant recipients. In this study they investigated and compared the pharmacokinetics of tacrolimus BID (n=62) and tacrolimus QD (n=67) formulations, given within 6–12 hours after transplantation (46). Mean $AUC_{0\text{-}24}$ of tacrolimus on day 1 after transplantation was approximately 50% lower for tacrolimus QD than tacrolimus BID at equivalent doses, but was comparable by day 14 and week 6, although the mean daily doses of tacrolimus QD doses were higher after day 1. In practice, to achieve similar tacrolimus exposure, the initial dose of tacrolimus QD would need to be higher than the total tacrolimus BID dose. T_{max} occurred later for tacrolimus QD compared with tacrolimus BID: day 1: 5.0 versus 2.9 hours; day 14: 2.6 versus 1.9 hours; week 6: 2.8 versus 1.8 hours, respectively. This reflects

the prolonged-release characteristics of the tacrolimus QD formulation. The reasons for the differences in the extent of absorption between the QD and BID formulations in the immediate post transplant period are not fully understood. Fischer et al demonstrated a good correlation between whole-blood tacrolimus trough levels and $AUC_{0\text{-}24}$ for both tacrolimus formulations. In clinical practice, for drug-level monitoring, the same recommended target levels can be applied for both formulations to ensure similar exposure. Close monitoring in the early post conversion period is recommended, because of the differences in the extent of absorption between the QD and BID formulations. Fischer et al also studied acute rejection, graft and patient survival (1-year follow-up) as secondary endpoint. They found no significant differences in these parameters between the tacrolimus BID and QD treated patients. Frequently reported adverse events during the study were renal impairment, diabetes mellitus, vascular hypertensive disorders, hyperglycaemic conditions. There were no differences between the two groups for these adverse effects. Some studies that investigated tacrolimus BID versus Tacrolimus QD, but not in liver transplant recipients, suggest that tacrolimus QD is associated with lower peak levels than tacrolimus BID. These lower peak levels may lead to a better control of glycaemic metabolism (47). However, these studies found no reduction of cardiovascular adverse events, insulin-dependent diabetes, lipid-lowering agents and antihypertensive drugs per patient. In conclusion, the tacrolimus QD formulation has several advantages compared to the BID formulation. A once daily regimen may increase patient adherence and quality of life. Mean AUC of tacrolimus was different for the two formulations early after transplantation, but were comparable from day 14 and later on. Since no differences in adverse effects between the two formulations were found in previous studies, we recommend conversion from the tacrolimus BID to tacrolimus QD formulation in stable LT patients, to increase adherence and quality of life.

Adverse effects of CNIs

The use of CNIs may have a negative influence on kidney function; induce diabetes mellitus and hypertension, however depending on type of CNI, dosing regimens and concomitant immunosuppressive medication. Nephrotoxicity is one of the most serious complications of CNIs (48). Apart from intestinal transplants, liver transplant recipients have the highest five-year incidence of chronic renal failure (CRF) of any non-renal solid organ transplant recipient; additionally, the risk of death is at least fourfold higher in patients who develop CRF (49). Five years after liver transplantation up to 18% of the patients have chronic renal failure. Diabetes mellitus, hypertension and hepatitis C virus infection are independent risk factors for renal failure (50). A recent study in 405 patients, who underwent a liver transplantation between 1986 and 2008 at the Erasmus Medical center (EMC) and were alive at one year showed that stage 3 chronic kidney disease (CKD-III; GFR < 60 ml/min) developed in 168 patients (43%). The one- and five

year cumulative incidence of CKD-III was 29.7 ± 2.3% and 41.1 ± 2.6% respectively. End-stage kidney disease (ESKD) requiring either dialysis or kidney transplantation developed in 11 patients. The cumulative incidence of ESKD at ten years was 4.3 ± 1.7%. The one-, two- and three year cumulative incidence of CKD-III in the 194 patients using tacrolimus was 21.0 ± 0.03, 24.5 ± 0.03 and 26.4 ± 0.03 respectively (unpublished data, Azimpour, Metselaar et al., 2009). The recent introduction of the Model for endstage liver disease (MELD) to allocate donor livers in the Netherlands increases the probability that recipients have pre-transplant renal dysfunction that can further deteriorate with the use of CNIs after transplantation. Moreover, the long-term use of immunosuppressive agents has been associated with an increased risk of developing de novo cancer. A recent study in 385 patients transplanted at the Erasmus MC, Rotterdam demonstrated a 2.2-fold higher incidence of de novo cancer as compared to the general population. The cumulative incidences at 1, 5, 10 and 15 years after liver transplantation were 2.9%, 10.5%, 19.4% and 33.6% respectively (51). These data are in line with a population-based study from Groningen with an overall relative risk as compared with the general population of 4.3 (95% confidence interval 2.4–7.1). Multivariate analysis showed that an age > 40 year and pre-transplant use of immunosuppression were significant risk factors (52). Furthermore, CNIs are related with new-onset diabetes mellitus after transplantation (NODAT). NODAT has been reported to occur in 2.5% to 25% of liver transplant recipients, and 2% to 53% of all solid organ transplants (53,54). The variation in the reported incidence may be due in part to the lack of a universal agreement on the definition of NODAT, the duration of follow-up, and the presence of modifiable and non-modifiable risks factors. Current WHO and American Diabetes Association (ADA) guidelines for the diagnosis of pre-diabetic states (IFG and IGT) and diabetes mellitus are outlined by Pham et al (55). The incidence of NODAT after liver transplantation has also been found to be higher in tacrolimus-treated versus cyclosporin-treated patients at one year post transplant. In a large randomized trial involving more than 500 liver transplant recipients, NODAT occurred in 26.6% versus 16.1% of patients receiving tacrolimus and cyclosporin immunosuppressive therapy, respectively (56). Other risk factors for NODAT reported in literature are age, weight, ethnicity, family history and hepatitis C (57). In HCV-infected liver recipients, the prevalence of post-transplant diabetes ranges between 40% and 60% (58–60). Despite of the incidence of adverse effects such as chronic renal failure, hypertension, de novo malignancy and new-onset diabetes mellitus, tacrolimus still remains the cornerstone of preventing rejection after liver transplantation. The occurrence of these adverse effects, has led to a focus shift from acute cellular rejection and short-term post transplant survival to long-term management of complications. Furthermore, strategies to minimize CNI exposure have been developed.

CNI optimization strategies

Switch to CNI-free regimen when CNI-toxicity has evolved.

The impact of CNIs on renal function after liver transplantation has led to a number of strategies to minimize CNI exposure (61). One approach is to switch to a calcineurin inhibitor-free regimen when CNI toxicity, such as renal dysfunction, has evolved. Several studies have evaluated CNI conversion to the Mammalian target of rapamycin (mTOR) inhibitors, such as sirolimus and everolimus. All these studies show that switching to sirolimus or everolimus improved to some extent renal function, especially early after conversion. However, accumulated literature shows that late mTOR conversion does not improve long-term renal outcome in liver transplantation. Moreover, conversion to a calcineurin inhibitor-free regimen was associated with rejections up to 35%. Because of serious side-effects a return to the calcineurin inhibitor occurred in up to 30% of the patients on mTOR inhibitors (62–69).

Start with mTOR inhibitor in combination with other IS drugs directly post-LT

Another approach to tackle the calcineurin inhibitor-associated problems after liver transplantation might be to completely avoid these drugs and start from the time of transplantation with mTOR inhibitors in combination with other immunosuppressive drugs. However, this approach will lead to a higher rate of rejection and major concerns consist about hepatic artery thrombosis, delayed wound healing, thrombocytopenia and proteinuria in the mTOR treated patients (70–73). *De novo* sirolimus is therefore not recommended in liver transplantation in the USA due to this supposed increased risk of hepatic artery and portal vein thrombosis and increased mortality. In Europe, sirolimus is approved to be used in renal transplant recipients.

Delayed introduction of reduced-dose tacrolimus under protection of MMF and dacluzimab

A third approach is delayed introduction of reduced-dose tacrolimus under the protection of mycophenolate mofetil (MMF) and dacluzimab. A recent study demonstrated that postponing introduction of tacrolimus was associated with less impairment of renal function without an increased frequency of rejection or graft loss. However, mean change from baseline in calculated creatinine clearance was still 13.6 ml/min 52 weeks after transplantation. Moreover, the CNI exposure in the control group was rather high as compared to current practice in liver transplantation, overestimating the protective effect of delayed introduction of tacrolimus (7). Another randomized controlled trial comparing dacluzimab, delayed low-dose tacrolimus (target trough level 4–8 ng/ml) to standard dose tacrolimus (trough level 10–15 ng/ml) and MMF showed comparable results (6). Delayed low-dose tacrolimus preserved early renal function without the cost

of increased rejection. At six months after transplantation the difference in cGFR (MDRD) was small, but statistically significant (75.4 vs 69.5 ml/min/1.73 m²). However, the long-term benefits of this approach remain to be established.

Switch to CNI-free regimen early after LT

A fourth approach is to switch to a calcineurin inhibitor-free regimen early after transplantation. This approach has three potential advantages. It prevents the supposed risk of hepatic artery thrombosis and impaired wound healing of mTOR inhibitors. Moreover, conversion is initiated before unrecoverable renal injury of calcineurin inhibitors has occurred. Currently, several international studies are running evaluating the efficacy and safety of mTOR inhibitors with corticosteroids in combination with elimination of calcineurin inhibitor in *de novo* liver transplant recipients compared to standard immunosuppressive therapy. Most of these studies focus on renal function one year after liver transplantation. One multicenter study has recurrence of hepatocellular carcinoma as primary endpoint. The complete withdrawal of CNIs in these studies warrants higher levels of mTOR inhibitors to avoid under-immunosuppression with an increased risk of allograft rejection. As a consequence of this approach a higher discontinuation rate due to side-effects can be expected, as is demonstrated in the late conversion studies. Moreover, a recent FDA alert notified healthcare professionals of clinical data that suggest increased mortality in stable liver transplant patients after conversion from a CNI-based immunosuppressive regimen to sirolimus. Moreover, the overall treatment failure rate at one year, defined as the occurrence of acute rejection or premature discontinuation for any reason, for the intent-to-treat population was significantly higher for the cohort of stable liver transplant patients converted to sirolimus compared to the cohort that continued CNI. (http://www.fda.gov/drugs/DrugSafety/default.htm). All these data support a different approach, namely to combine tacrolimus and sirolimus at low dosages in the early phase after liver transplantation and use their potential synergism, as is suggested in several preclinical studies (74,75). It is expected that this approach, combining both drugs at lower dosages, equals the protective capacity of tacrolimus against allograft rejection, preserve renal function in a significant way and has an acceptable tolerability profile. Moreover, it can be postulated that the incidence of *de novo* malignancy after transplantation will be reduced as the use of mTOR inhibitors is associated with a lower risk of developing cancer (76–78). These anti-neoplastic properties of mTOR inhibitors are further supported in experimental studies (79–81). An uncontrolled study using low-dose tacrolimus and sirolimus in 56 liver transplant recipients showed an excellent efficacy with a rejection rate of 14%. The target trough levels of tacrolimus were 5 ng/ml and of sirolimus 7 ng/ml. Corticosteroids were weaned off at 3–6 months. Renal function in these patients represented a 30% improvement as compared to historical controls with normal dose tacrolimus (82). However, the potential benefit of combining

low-dose tacrolimus and sirolimus must be verified in randomized controlled trials. In conclusion, the impact of CNIs on renal function after liver transplantation has led to a number of strategies to minimize CNI exposure. A first approach is to switch to CNI-free regimen when CNI-toxicity has evolved. Another approach is to start with mTOR inhibitor in combination with other IS drugs directly post-LT. A third approach is delayed introduction of reduced-dose tacrolimus under the protection of mycophenolate mofetil (MMF) and dacluzimab. A fourth approach is to switch to a calcineurin inhibitor-free regimen early after transplantation. However, long term benefits of these approaches have to be established.

Practice points

- CNIs are still the cornerstone of immunosuppresion after liver transplantation.
- Tacrolimus still remains the first-choice immunosuppressant in liver transplant recipients, also in HCV-infected patients.
- CNIs have a narrow therapeutic window necessitating close drug monitoring.
- Conversion from the tacrolimus BID to tacrolimus QD formulation in stable LT patients is safe and will increase adherence and quality of life.
- Strategies to minimize CNI exposure will improve outcome after liver transplantation.

Research agenda

- Because of adverse effects of tacrolimus, CNI minimization strategies need further exploration to improve long term outcome after liver transplantation.
- Whether the use of mTOR inhibitors will reduce the incidence of malignancy after liver transplantation needs to be studied.
- Genetic differences in CYP3A enzymes and ethnic background could influence the tacrolimus metabolism. Whether these differences have clinical implications needs to be studied in larger cohorts.

Conflict of interest

H.J. Metselaar received several research, travel and lecturer grants from Astellas Pharma, Novartis and Biotest GmbH in the past five years.

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CHAPTER 8

Human liver graft-derived mesenchymal stromal cells potently suppress allo-reactive T-cell responses

Emmy L.D. de Mare-Bredemeijer[1]
Shanta Mancham[1]
Monique M.A. Verstegen[2]
Petra E. de Ruiter [2]
Rogier van Gent[1]
David O'Neill[4]
Hugo W. Tilanus[2]
Herold J. Metselaar[1]
Jeroen de Jonge[2]
Jaap Kwekkeboom[1]
Sean R.R. Hall[2,3]*
and Luc J.W. van der Laan[2]*

* Shared last authorship
1 Department of Gastroenterology & Hepatology
2 Department of Surgery, Erasmus MC-University Medical Center, Rotterdam, The Netherlands
3 Division of Thoracic Surgery, University Hospital Bern, Switzerland
4 Neostem Inc., New York, USA

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ABSTRACT

After organ transplantation, recipient T cells contribute to graft rejection. Mesenchymal stromal cells from the bone marrow (BM-MSCs) are known to suppress allogeneic T-cell responses, suggesting a possible clinical application of MSCs in organ transplantation. Human liver grafts harbor resident populations of MSCs (L-MSCs). We aimed to determine the immunosuppressive effects of these graft-derived MSCs on allogeneic T-cell responses and to compare these with the effects of BM-MSCs. BM-MSCs were harvested from aspirates and L-MSCs from liver graft perfusates. We cultured them for 21 days and compared their suppressive effects with the effects of BM-MSCs on allogeneic T-cell responses. Proliferation, cytotoxic degranulation and IFN-γ production of allo-reactive T cells were more potently suppressed by L-MSCs than BM-MSCs. Suppression was mediated by both cell-cell contact and secreted factors. In addition, L-MSCs showed *ex vivo* a higher expression of PD-L1 than BM-MSCs, which was associated with inhibition of T-cell proliferation and cytotoxic degranulation in vitro. Blocking PD-L1 partly abrogated the inhibition of cytotoxic degranulation by L-MSCs. In addition, blocking IDO partly abrogated the inhibitive effects of L-MSCs, but not BM-MSCs, on T-cell proliferation. In conclusion, liver graft-derived MSCs suppress allogeneic T-cell responses stronger than BM-MSCs, which may be related to in situ priming and mobilization from the graft. These graft-derived MSCs may therefore be relevant in transplantation by promoting allo-hyporesponsiveness.

INTRODUCTION

Mesenchymal stromal cells (MSCs) are rare, non-hematopoietic cells and reside in the bone marrow (BM) cavity. They are characterized by their ability to produce colony forming unit-fibroblast (CFU-F); support of the hematopoietic microenvironment; promotion of bone formation and adherence to plastic in vitro (1-3). Besides their presence in the BM, MSC-like cells are present throughout the body, occupying a perivascular location [4, 5] and are critically involved in maintaining tissue homeostasis via anti-apoptotic and tissue-supporting properties (6-8).

It has been demonstrated that resting or naive MSCs are inherently capable of suppressing T-cell responses (9-11). Bone marrow MSCs (BM-MSCs) can suppress recipient allo-reactive T-cell responses and thereby prevent graft rejection (12), suggesting a possible clinical application of MSCs in organ transplantation. Human liver grafts also contain MSCs (L-MSCs) [13, 14] and these are mobilized from the liver graft during the transplantation procedure. MSCs express several cell surface receptors that enable them to sense the microenvironment and alter their phenotype accordingly [6, 15]. Many studies have shown that it is the nature of the environmental cues that dictates the plasticity and - in the end - immunosuppressive capacity of MSCs (14-27). Most of the data that support this concept of MSC 'mobilization' and immune 'priming' are based on experimental mouse studies. But because humans and mice differ in immunomodulatory pathways used by MSCs, there is an urgent need to assess the immunosuppressive function of human MSCs in the clinical setting of liver transplantation (16).

In addition, it is still unknown whether human graft-derived MSCs are able to suppress allo-reactive T-cell responses as potently as their BM counterparts. Although the liver is an immunologically tolerogenic organ (17-19), it is unknown whether MSCs in healthy liver contribute to this tolerogenicity. The organ donation process is associated with a wide range of hemodynamic and inflammatory changes throughout the body [20, 21] that may well affect the inherent immunosuppressive properties of MSCs in the liver graft. Inflammation affects pathways that can inhibit T-cell responses, such as the PD-L1/PD-1 pathway. PD-1 is an inhibitory receptor expressed on T cells, which inhibits T-cell responses after interaction with its ligand PD-L1 [22, 23], which is expressed on antigen presenting cells and MSCs. PD-L1 is known to be up-regulated after exposure to inflammatory cytokines, such as IFN-γ (24). Another factor that is up-regulated in MSCs in response to IFN-γ is indoleamine 2,3-dioxygenase (IDO) [25, 26], which has been associated with the immunosuppressive capacity of MSCs. Therefore, these mechanisms may well contribute to the inhibition of allo-reactive T cells by MSCs after liver transplantation. In addition, soluble factors secreted by MSCs, such as transforming growth factor-beta (TGF-β), hepatocyte growth factor (HGF) (27) and prostaglandin E2 (PGE2) (9) may contribute to their capacity to inhibit allo-reactive T-cell responses as well.

In the present study, we aimed to assess whether graft-derived MSCs can suppress allo-reactive T-cell responses and if so, whether they suppress more potently than their BM counterparts. To assess this, we isolated L-MSCs from liver perfusates obtained from donor livers and added these cells to mixed lymphocyte reactions. We measured proliferation and effector function of allo-reactive T cells in these co-cultures in the presence or absence of L-MSCs. These outcomes were compared with those of co-cultures with BM-MSCs.

MATERIALS AND METHODS

Ethics Statement

Liver perfusates and splenocytes were obtained from deceased donors following organ donation. The use of all human materials was approved by the Medical Ethical Committee of the Erasmus MC-University Medical Center Rotterdam and the study was performed in accordance with the amended Declaration of Helsinki.

Isolation and culture of MSCs

BM-MSCs were harvested from aspirated bone marrow as previously described (28). L-MSCs were isolated from perfusates of human liver grafts. Liver perfusates were collected from liver graft donors during organ donation procedure, by flushing the transplant liver with 1 liter of University of Wisconsin (UW) preservation solution followed by a second flush with 400 ml human albumin during the back table bench procedure, prior to implantation, as previously described (14). Liver perfusates were collected and centrifuged (1500 rpm, 4°C; 10 minutes) to pellet cells. The cell pellet was resuspended in PBS and mononuclear cells (MNCs) were isolated using Ficoll Hypaque density gradient separation. MNCs were counted and resuspended in culture medium consisting of alpha-MEM/GLUTAMAX (Gibco), 2% FBS, 1% antibiotic-antimycotic (Gibco) supplemented with 20 ng/ml of recombinant human (rh)EGF (eBioscience) and 10 ng/ml of rhFGF2 (eBioscience), plated at $1.0x10^5$ cells/cm^2 in 10 cm dishes and cultured at 37°C, 5% CO_2. After 3 days, the nonadherent fraction was removed and medium was replaced by fresh medium. Cells were cultured until colonies became confluent and were harvested using a nonenzymatic solution (TrypLE, Gibco) and further expanded for later use. Early passage cells from bone marrow were cultured and expanded in the same culture conditions as described for L-MSCs.

Immunophenotypic profile of MSCs in liver perfusates and BM-MSCs

To detect the presence of mobilized MSCs and hematopoietic stem/progenitor cells (HSCs) in liver perfusates from deceased donors, MNCs collected from the liver perfusates, as

described above, were counted for viability and stained to detect and quantify MSCs. For HSCs, MNCs were stained with the following antibodies: lineage-FITC (BD Pharmingen), CD34-PE (BD Pharmingen), CD38-APC-Cy7 (eBioscience), CD45RA-PB (eBioscience), and CD90-APC (eBioscience). For MSCs, MNCs were stained with the following antibodies: CD45-PB (eBioscience), CD146-PE (eBioscience), CD44-APC-Cy7 (eBioscience), CD73-APC (eBioscience), CD105-PE (eBioscience), CD90-FITC (eBioscience), CD14-PB (eBioscience), CD19-APC-Cy7 (eBioscience), HLA-ABC-FITC (eBioscience), and HLA-DR-APC (eBioscience). The same antibodies were used to stain BM-MSCs to compare the immunophenotypic profile of L-MSCs and BM-MSCs. The immunophenotypic profile of L-MSCs and BM-MSCs was carried out on early passage (P3) cells. Prior to addition of antibodies, cells were treated with Fc block (Miltenyi). Cells were stained at 4°C in the dark for 30 minutes. Afterwards, cells were washed, centrifuged and resuspended in PBS prior to flow cytometric analysis. All cells were incubated with 7-AAD (eBioscience) to discriminate dead/dying cells and debris. Samples were collected and a minimum of 1 million cells were measured on a BD FACS Canto II and analyzed using FlowJo software (Tree Star). In the analyses, gates were based on fluorescence-minus-one controls.

Differentiation Assays

Early passage (P3) L-MSCs were placed in defined culture conditions and compared with early passage (P3) BM-MSCs. For osteogenic differentiation, cells were plated at 4000 cells/cm^2 per well in a 6-well dish (Corning) in regular culture medium as described above, and placed in a humidified chamber with 5% CO_2 at 37°C. After 24 hours, medium was changed to osteogenic induction medium (alpha-MEM/GLUTAMAX, 10% FBS, 1% Penicillin-streptomycin, 50 µM ascorbic acid (Sigma), 10 mM β-glycerophosphate (Sigma), 100 nM dexamethasone (Calbiochem)). To induce differentiation, half of the medium was replaced by fresh medium every 3 days for a total of 21 days. After 21 days, cell cultures were fixed with 4% paraformaldehyde (Sigma) for 15 minutes, washed twice with PBS and stained with 40 mM Alizarin Red S stain at pH = 4.1 (Sigma) for 30 minutes to detect mineralization.

For adipogenic induction, 1x10^5 cells were plated per well in a 6-well dish in regular culture medium and placed in a humidified chamber with 5% CO_2 at 37°C. After 24 hours, the wells were washed with PBS and fresh adipogenic maintenance medium (DMEM/Low glucose (Gibco), 10 µg/mL human insulin (Invitrogen), 10% FBS, 1% Penicillin/Streptomycin) was added. After 3 days, the medium was changed to adipogenic induction medium (DMEM/Low glucose, 10 µg/mL human insulin, 100 µM indomethacin (Sigma), 0.5 mM IBMX (Sigma), and 1 µM dexamethasone). After 3 days, medium was changed back to adipogenic maintenance medium. After an additional 2 rounds of maintenance-induction media changes, cells were incubated for an additional 7 days in adipogenic

maintenance medium. After this, cells were fixed with 4% paraformaldehyde and stained with Oil Red O (Sigma) to detect formation of lipid droplets.

Immune priming of MSCs with IFN-γ plus TNF-α

Passage 3 culture expanded L-MSCs and BM-MSCs were plated at $1.0x10^5$ cells per well in a 6-well dish in regular culture medium. Cells were grown for 48 hours and afterwards were growth arrested in serum-free media (RPMI 1640, Gibco) for 24 hours. Following this, cells were treated with 10 ng/mL of rhTNF-α and 10 ng/ml of rhIFN-γ. After 24 hours, the cell media were collected, frozen down and stored at -80°C until further use. The cells were stained with the following primary fluorescently conjugated antibodies: PD-L1-APC (eBioscience), CD73-FITC (eBioscience), CD45-PB (eBioscience), HLA-DR-PE (eBioscience) in the presence of Fc blocking reagent (Miltenyi) at 4°C in the dark for 30 minutes. Cells were analyzed using a BD FACS Canto II Flow cytometer (BD Biosciences, San Jose, CA). 7-AAD was included to discriminate dead/dying cells and debris. A minimum of 10,000 events were collected and analyzed using FlowJo software (Tree Star).

Mixed lymphocyte reactions (MLR) in the presence or absence of MSCs

To test the suppressive capacity of MSCs on allogeneic T-cell responses, we performed mixed lymphocyte reactions (MLRs), in which we stimulated CFSE-labeled peripheral blood mononuclear cells (PBMCs) with allogeneic CD40-ligand stimulated (CD40)-B cells in the presence or absence of L-MSCs or BM-MSCs. PBMCs were isolated from blood of healthy blood bank donors using Ficoll Hypaque density gradient centrifugation and cryopreserved at -135°C until further use. CD40-B cells, which were used as allogeneic stimulator cells, were expanded from organ donor splenocytes, as previously described (29). For different experiments different responder and stimulator combinations were used. Both PBMCs and CD40-B cells were thawed and recuperated overnight in B-cell medium (IMDM + 10% human serum + 1% Penicillin/Streptomycin (Gibco) + 1% Insulin/Transferrin/Selenium (Gibco)) at 37°C and 5% CO_2. PBMCs were labeled with 0.5 µM CFSE (Invitrogen, Paisley, UK) according to the manufacturer's instructions and CFSE-labeled PBMCs ($1x10^5$) were stimulated with $2x10^5$ irradiated (30 Gy) donor CD40-B cells in 96-wells U-bottom plates in a final volume of 250 µl B-cell medium. In separate wells, $1x10^4$ or $2x10^4$ irradiated (30 Gy) liver graft-derived L-MSCs or BM-MSCs were added to the co-cultures. PBMCs stimulated with 5 µg/ml Phytohemagglutinin (PHA) (Murex) were included as positive controls to assess their proliferative capacity. Each culture condition was performed in duplicate.

In separate experiments, we studied the role of PD-L1, IDO and PGE2 in inhibition of allogeneic T-cell responses by MSCs by adding anti-PD-L1 mAb (10 µg/ml; eBioscience) to block PD-L1; 1Methyl-DL-tryptophan (1MT; 250 µM; Sigma-Aldrich) to block IDO; EP1-3 receptor blockers (10 µM; AH6809, ITK Diagnostics) and EP4 receptor blocker (20 µM;

AH23848, Sigma-Aldrich) to block PGE2. In addition, to test the role of PGE2, L-MSCs and BM-MSCs were pre-treated overnight with 5 μM of Indomethacin (I7378, Sigma Aldrich). Indomethacin (5 μM) was also present during the 5 days of co-culturing.

After 5 days of culture at 37°C and 5% CO_2, cell-free supernatant was collected, frozen and stored at -20°C for later analysis, and cells were stained for cell viability, using the LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen), according to the manufacturer's protocol. Cells were then stained with anti-CD3-PerCP-Cy5.5 (BD Biosciences), anti-CD4-APC-H7 (BD Biosciences), anti-CD8-efluor450 (eBioscience) to distinguish T-cell subsets and anti-CD19-horizonV500 (BD Biosciences) to exclude B cells from analysis. Cytotoxic degranulation was detected by addition of CD107a-APC (eBioscience) during the last 15 hours of the co-cultures. Cells were analyzed for proliferation, using CFSE-dilution patterns, and for phenotype on a BD FACS Canto II Flow cytometer (BD Biosciences, San Jose, CA). For analysis of phenotypic markers we used FACS Diva software (Becton Dickinson) and precursor frequencies (PF) of allo-reactive $CD4^+$ and $CD8^+$ T cells were calculated using ModFit LT® software (Verity Software House, USA), as previously described (29). From duplicate assays, average precursor frequencies were calculated.

IFN-γ production was measured in the culture supernatants by standard enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Human IFNγ CytoSetTM, Invitrogen). TNF-α production was also measured in the culture supernatants by ELISA (human TNF-α Ready-Set-Go!, eBioscience)

Polymerase Chain Reaction (PCR)

Early passage (P3) liver perfusate-derived and bone marrow-derived MSCs were plated at 1.0×10^5 cells per well in a 6-well dish (Corning) in full growth medium. After 24 hours cells were growth arrested in serum free media (RPMI) for 24 hours. Following this, 700 μl of Qiazol lysis buffer (Qiagen) was added to each well and cells were harvested. RNA was isolated using a Qiagen miRNeasy mini kit (Qiagen, Venlo, the Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). A total of 300 ng was used to make cDNA using an iScript cDNA synthesis kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA) and 15 ng cDNA was used per real-time quantitative PCR (RT-qPCR) reaction. The expression of prostaglandin-endoperoxide synthase 1 and 2 (PTGS1 and PTGS2) was quantified using RT-qPCR with a SensiMix Plus SYBR Kit (BioLine, London, UK) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a household gene for normalization of gene expression. RT-qPCR was performed using the following primers; PTGS1-forward CGCCAGTGAATCCCTGTTGTT; PTGS1-reverse AAGGTGGCATTGACAAACTCC; PTGS2-forward CTGGCGCTCAGCCATACAG, PTGS2-reverse CGCACTTATACTGGTCAAATCCC. GAPDH-forward: AAGGTCGGAGTCAACGGATTT, GAPDH-reverse: ACCAGAGTTAAAAGCAGCCCTG. The fold change in mRNA was determined using the ΔCt method.

Statistics

Data were analyzed with GraphPad Prism 5.0 software and expressed as mean ± SEM. To test whether data were normally distributed the Kolmogorov-Smirnov test was used. Differences between groups were analyzed using the paired t-test (normally distributed paired data), Wilcoxon signed rank test (non-normally distributed paired data) or Mann Whitney test (unpaired data). One-sample t-test was performed to test differences between experimental conditions and control conditions consisting of one sample. P-values < 0.05 were considered significant.

RESULTS

Immunophenotypic and differentiation potential of MSC-like cells mobilized from liver grafts are similar to those of BM-MSCs

To explore whether MSCs mobilize from human liver grafts after tissue injury *in vivo* we collected liver graft perfusates during flushing of liver grafts at time of transplantation. These livers have been exposed to hemodynamic, ischemic and inflammatory changes during the donation process. From these liver perfusates, we recovered a population of cells that adhered to plastic and were amenable to expansion, like BM-MSCs (Figure 1A and B). In addition, these graft-derived cells could well differentiate into adipocytes and osteocytes (Supplemental Figure 1). Likely these cells originate from the graft parenchyma and not from residual donor blood, as MSCs could not be recovered from donor blood cells [14, 30]. To confirm whether these graft-derived cells were of mesenchymal origin we compared their phenotype with that of BM-MSCs. This analysis revealed that liver perfusate-derived cells lacked the hematopoietic markers CD45, HLA-DR, CD14 and CD19 (representative flow cytometric plots in Figure 1C) while they expressed the common mesenchymal markers CD146, CD44, CD73, CD90 and CD105 (Figure 1D). This phenotype was similar to that of BM-MSCs (Figure 1C and D). Combined, these data suggest that MSC-like cells in perfusates represent genuine graft-derived liver MSCs.

L-MSCs suppress allo-reactive T cells better than BM-MSCs

Previously, it has been shown that BM-MSCs can suppress proliferation of allo-primed T cells (9). We compared the suppressive capacity of L-MSCs with that of BM-MSCs in mixed lymphocyte reactions (MLRs) in which CFSE-labeled PBMCs were stimulated with allogeneic splenocyte-derived CD40-B cells as strong antigen presenting cells, as described previously (29). The level of T-cell proliferation was determined using CFSE-dilution patterns, from which we calculated precursor frequencies (PF) of responding T cells, as shown in Figure 2A. We found that both $CD4^+$ and $CD8^+$ T cells proliferated in response to stimulation with allogeneic CD40-B cells over a 5-day period. Precursor frequencies in

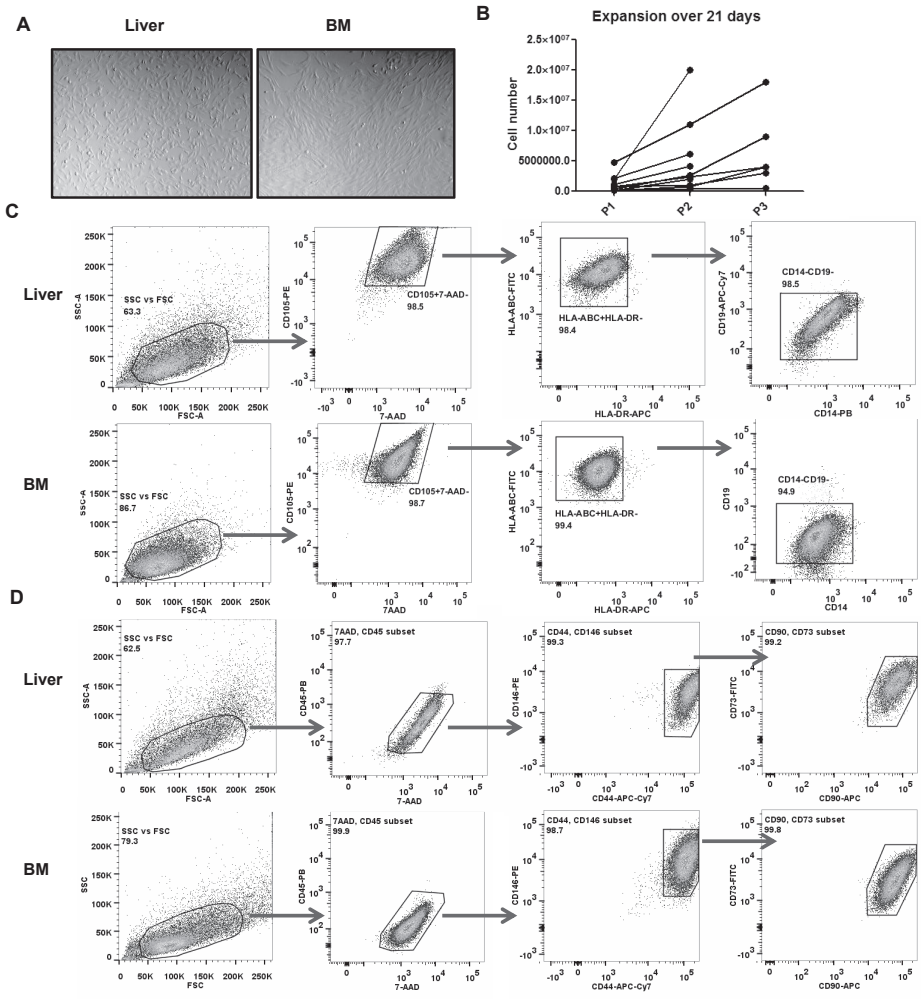

Figure 1.
Detection of mesenchymal stromal (MSC)-like cells in the perfusates from human liver grafts. (A) Representative phase contrast images of plastic adherent cells recovered from the mononuclear cell fraction of liver perfusates from liver grafts and from bone marrow (BM). (B) Expansion of plastic adherent cells obtained from the perfusates of liver grafts over the course of 21 days. P1-3: passage 1-3. (C) Representative multicolour flow cytometric plots demonstrating that culture expanded plastic adherent cells recovered from liver perfusates lacked the hematopoietic markers CD45, HLA-DR, CD14 and CD19. (D) Representative multicolour flow cytometric plots demonstrating that the immunophenotypic profile of culture expanded plastic adherent cells recovered from liver perfusates was similar to that of BM-MSCs, based on CD45-CD146$^+$CD44$^+$CD73$^+$CD90$^+$. Gates are based on fluorescence minus one in control conditions.

the control conditions varied from ~2-10%, because different responder and stimulator cells combinations were used for different experiments. To test the suppressive capacity of L-MSCs and BM-MSCs, we added L-MSCs or BM-MSCs to the co-cultures at an MSC/

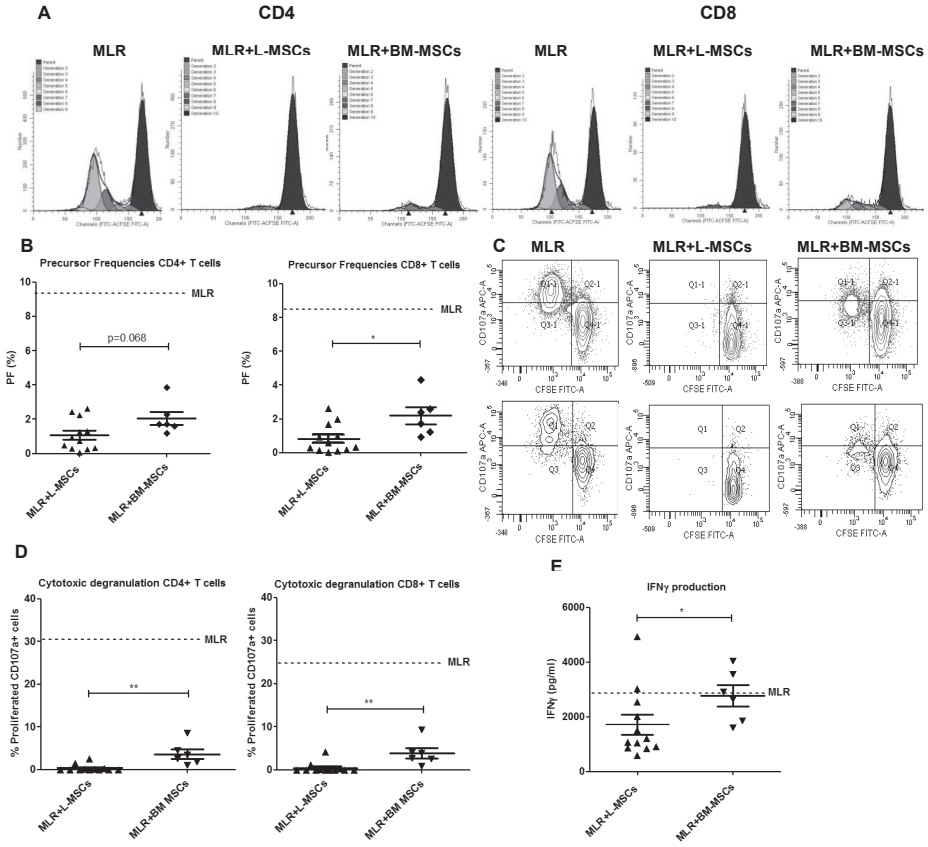

Figure 2.

Graft-derived L-MSCs suppress allo-reactive T-cell responses stronger than BM-MSCs. CFSE-labeled PBMCs were cultured for 5 days with allogeneic splenocyte-derived CD40-ligand stimulated B cells (CD40-B cells). Mixed lymphocyte reactions (MLR) were performed in the presence and absence of either L-MSCs (n = 12) or BM-MSCs (n=6). CFSE-dilution patterns of $CD4^+$ and $CD8^+$ T cells were analyzed and precursor frequencies (PF) were calculated with ModFit® software. (A) Representative ModFit-derived CFSE-dilution patterns of $CD4^+$ (left panel) and $CD8^+$ (right panel) T cells after 5 days stimulation with allogeneic CD40-B cells with and without MSCs. (B) Scatter dot plots showing precursor frequencies of allo-reactive $CD4^+$ and $CD8^+$ T cells in the MLR without MSCs (control MLR, dotted line) and in the MLRs with L-MSCs versus BM-MSCs. Data depicted as mean ± SEM. (C) In parallel, flow cytometric analysis of CD107a (y-axis) on $CD4^+$ (upper panel) and $CD8^+$ (lower panel) T cells in the MLRs was performed. Representative flow cytometric plots are shown from control MLR, MLRs with L-MSCs and MLRs with BM-MSCs. (D) Scatter dot plots showing % proliferated $CD107a^+$ $CD4^+$ and $CD8^+$ T cells in the control MLR (dotted line) and in the MLRs with L-MSCs versus BM-MSCs. Data depicted as mean ± SEM. (E) In parallel, samples of the same PBMCs were analyzed for IFN-γ production upon stimulation with CD40-B cells. Five days after beginning the MLR, supernatants were harvested and IFN-γ levels were measured using ELISA. Scatter dot plots showing IFN-γ production in MLR (dotted line) and in MLRs with L-MSCs versus BM-MSCs. Differences between suppressive effects of L-MSCs versus BM-MSCs were tested using Mann Whitney test. * $p<0.05$ ** $p<0.01$ *** $p<0.001$

PBMC ratio of 1:10 or 1:5. With addition of L- MSCs (n =12, different donors) at ratio 1:5 proliferation of allo-reactive $CD4^+$ T cells was nine-fold lower and of $CD8^+$ T cells ten-fold lower than in control culture without L-MSCs. This difference was significantly different ($p<0.0001$). The anti-proliferative effect of BM-MSCs (n=6, different donors) was also evident, causing a four-fold lower proliferation of both $CD4^+$ and $CD8^+$ T cells than in control cultures without BM-MSCs ($p<0.0001$). Interestingly, L-MSCs suppressed $CD8^+$ T cell proliferation significantly better than BM-MSCs (Figure 2B; $p<0.05$). This superior suppressive effect of L-MSCs was also observed for $CD4^+$ T cells, although not statistically significantly (Figure 2B, $p=0.068$). With the addition of MSCs at MSC/PBMC ratio 1:10 we found the same trend towards a stronger anti-proliferative effect of L-MSCs than BM-MSCs, although the suppression was less strong and differences between L-MSCs and BM-MSCs were stronger at ratio 1:5. Apparently there is a dose-dependent inhibitive effect of MSCs on T-cell proliferation. In addition, L-MSCs inhibited cytotoxic degranulation, as measured with surface expression of CD107a on proliferated T cells, of both allo-reactive $CD4^+$ and $CD8^+$ T cells significantly better than BM-MSCs ($p<0.01$, Figure 2C, D). Finally, L-MSCs reduced IFN-γ production significantly ($p<0.01$) while BM-MSCs did not. This difference between BM-MSCs and L-MSCs was statistically significant (figure 2E, $p<0.05$). TNF-α production was strongly inhibited by both L-MSCs and BM-MSCs with no significant difference between L-MSCs and BM-MSCs (data not shown).

Soluble factors secreted by L-MSCs suppress $CD4^+$, but not $CD8^+$, T-cell proliferation and $CD4^+$ and $CD8^+$ cytotoxic degranulation

To assess whether the inhibition of L-MSCs on T-cell responses were cell-cell contact-independent, MLRs were performed in the presence of conditioned medium (CM) derived from MSC-cultures. We found that the CM from L-MSCs suppressed the proliferation of allo-reactive $CD4^+$ T cells, while CM from BM-MSCs did not (Figure 3A, $p<0.001$). CM from L-MSCs also suppressed proliferation of $CD8^+$ T cells significantly ($p<0.01$), but this effect was not significantly different from the suppressive effects of CM from BM-MSCs (Figure 3A). In addition, CM from L-MSCs suppressed the cytotoxic degranulation capacity of allo-reactive $CD4^+$ T cells and $CD8^+$ T cells significantly better than CM from BM-MSCs (Figure 3B, $p<0.001$ and $p<0.01$ respectively). CM from L-MSCs was not able to suppress IFN-γ production or TNF-α production (data not shown). Taken together, these data suggest that the immunomodulatory potential of L-MSCs is at least partially mediated by secreted factors.

L-MSCs express higher levels of PD-L1 than BM-MSCs, which is associated with suppression of allo-reactive T-cell responses

We wondered which mechanism could cause the superior immunosuppressive capacity of L-MSCs in comparison with their BM-MSCs counterparts. An important co-inhibitory

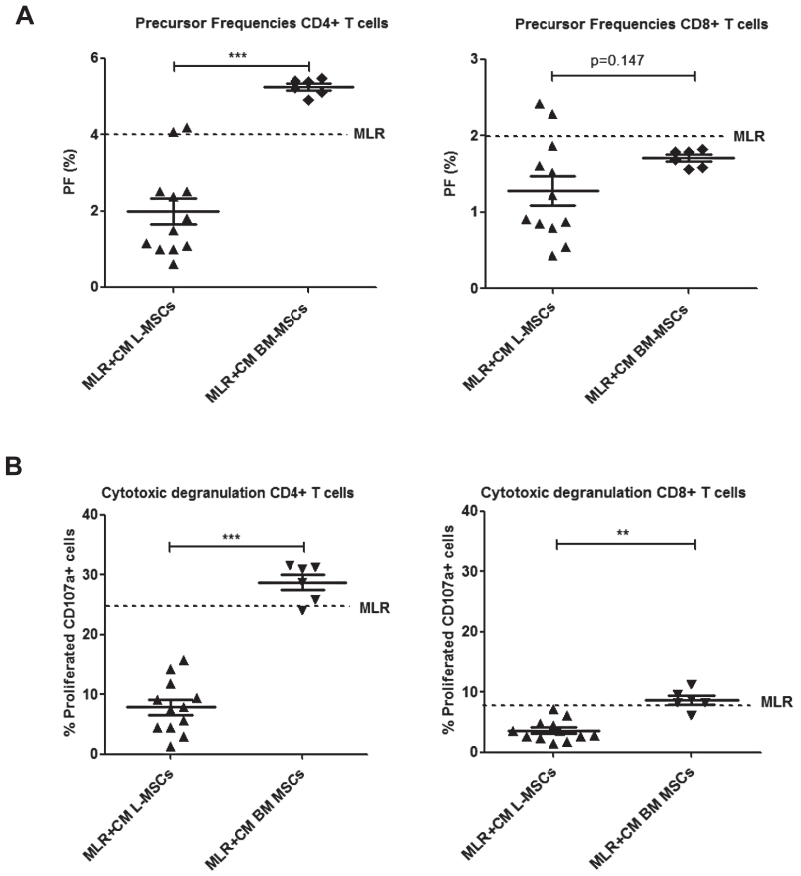

Figure 3.
Graft-derived L-MSCs suppress allo-reactive T-cell responses stronger by soluble factors than BM-MSCs. Supernatants from MSCs (conditioned medium (CM)) were added to the MLR and at the end of 5 days allo-reactive T-cell responses were measured. (A) Scatter dot plots showing precursor frequencies of allo-reactive $CD4^+$ and $CD8^+$ T cells in the MLR without CM (control MLR, dotted line) and in the MLRs with CM from L-MSCs versus CM from BM-MSCs. Data depicted as mean ± SEM. (B) Scatter dot plots showing % proliferated $CD107a^+$ $CD4^+$ and $CD8^+$ T cells in the control MLR (dotted line) and in the MLRs with CM L-MSCs versus CM BM-MSCs. Data depicted as mean ± SEM. * $p<0.05$ ** $p<0.01$ *** $p<0.001$

pathway in (allo-reactive) T-cell responses is the PD-1/PD-L1 pathway (31). We found that L-MSCs have higher baseline expression levels of PD-L1 than BM-MSCs (Figure 4A,C, $p<0.05$), which could be a reason for the higher suppressive capacity of L-MSCs. Pro-inflammatory cytokines have been described to up-regulate PD-L1. Since pro-inflammatory cytokines increase during the organ donation process, it is possible that during donation, MSCs in the liver become primed and thereby up-regulate PD-L1. To assess the effect of pro-inflammatory cytokine priming on PD-L1 expression and suppressive ca-

pacity, we exposed L-MSCs and BM-MSCs to TNF-α and IFN-γ *ex vivo* and measured their PD-L1 expression and suppressive function. After priming, we indeed found a strongly up-regulated PD-L1 expression on both L-MSCs and BM-MSCs (Figure 4B,C).

We wondered whether the superior suppressive effects of L-MSCs were mediated by their higher level of PD-L1 expression. We first looked at the correlation between PD-L1 expression on MSCs and the corresponding T-cell responses in the MLRs and found that

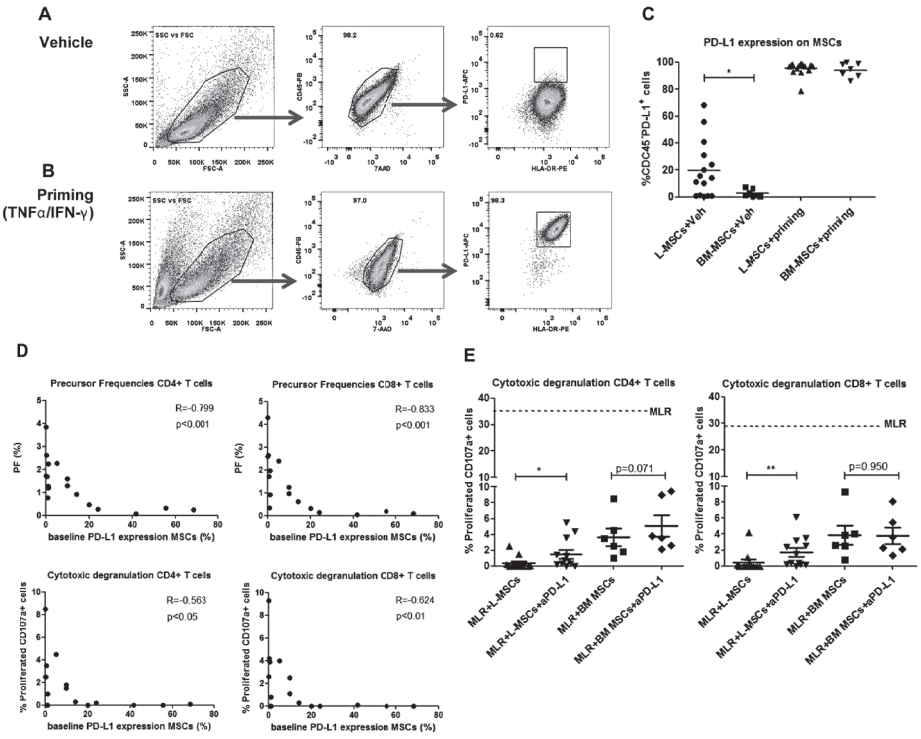

Figure 4.

Graft-derived L-MSCs express higher levels of PD-L1 than BM-MSCs; PD-L1 is up-regulated after *ex vivo* priming with pro-inflammatory cytokines and associated with suppression of allo-reactive T-cell responses. (A) Representative flow cytometric plots demonstrating baseline expression of PD-L1 on L-MSCs and BM-MSCs. (B) Representative flow cytometric plots demonstrating induced expression of PD-L1 following *ex vivo* priming with pro-inflammatory cytokines rh-IFN-γ/TNF-α at 10 ng/mL for 24 hours. (C) Scatter dot plot showing percentages of live CD45- cells expressing PD-L1 at baseline or following stimulation with rh-IFN-γ/TNFα, quantified using flow cytometry (L-MSCs, n=16; BM-MSCs, n=6). (D) Correlation between baseline PD-L1 expression on the MSCs added to the MLRs and precursor frequencies of $CD4^+$ and $CD8^+$ T cells in the MLRs and correlation between PD-L1 and cytotoxic degranulation in the MLRs. Correlation was tested using Spearman's rank correlation coefficient. To test whether the suppressive effects of L-MSCs and BM-MSCs could be abrogated by blocking PD-L1, a PD-L1 blocking antibody was added to the MLRs. (E) Scatter dot plots showing % proliferated $CD107a^+$ $CD4^+$ and $CD8^+$ T cells in the control MLR (dotted line); in the MLRs with L-MSCs versus L-MSCs+anti-PD-L1 and in the MLRs with BM-MSCs versus BM-MSCs+anti-PD-L1. Data depicted as mean ± SEM. Differences between MLRs with and without anti-PD-L1 were tested using paired t-test. * p<0.05 ** p<0.01 *** p<0.001

a higher PD-L1 expression on MSCs was strongly correlated with lower proliferation of both $CD4^+$ and $CD8^+$ T cells in the MLRs. In addition, higher PD-L1 expression levels were also strongly correlated with lower cytotoxic degranulation capacity of both $CD4^+$ and $CD8^+$ T cells in the MLRs (Figure 4D). We then added an anti-PD-L1 antibody to the MLRs to test whether blockade of the PD-1/PD-L1 interaction abrogated the suppressive effects of the L-MSCs. We found that anti-PD-L1 did not abrogate the suppressive effects of L-MSCs on proliferation and IFN-γ production or TNF-α production of T cells (data not shown), but significantly abrogated the effects on $CD4^+$ and $CD8^+$ T-cell cytotoxic degranulation (Figure 4E, $p<0.05$ and $p<0.01$ respectively).

Immunosuppressive effects of MSCs partly involves IDO, but not prostaglandin E2

In a previous study of our research group (14) we performed a gene expression profiling to compare the molecular phenotypes of L-MSCs and BM-MSCs. These data showed that L-MSCs and BM-MSCs were very similar, but a small number of genes was differentially expressed between L-MSCs and BM-MSCs. In the present study, we focused on the genes that were differentially expressed, to investigate whether these could provide insight into the differences in immunomodulatory effects of L-MSCs and BM-MSCs. One of the genes that were significantly differentially expressed was PTGS1, which encodes for Cyclooxygenase-1 (COX-1). COX-1 is the key enzyme in prostaglandin biosynthesis (32). Prostaglandins are produced by MSCs and have previously been described to inhibit T-cell responses (9). Since PTGS1 was 20-fold higher expressed in L-MSCs than BM-MSCs, differences in prostaglandin production between the two types of MSCs could well explain the immunomodulatory differences that we found. We first confirmed the difference in PTGS1 gene expression between L-MSCs and BM-MSCs using PCR (Figure 5A, $p<0.001$). We then investigated whether culturing MSCs in the presence of COX-1 inhibitor Indomethacin affected the immunomodulatory function of L-MSCs or BM-MSCs in the co-cultures with PBMCs. However, we found no effects of this (Figure 5B). In addition, we determined whether blockers of the prostaglandin receptors EP1,2,3 and 4 abrogated the inhibitive effects of CM from L-MSCs or BM-MSCs. However, we were not able to show higher rates of T-cell proliferation after addition of the blockers (Figure 5C).

Another suppressive mechanism described for MSCs is the production and activity of indoleamine 2,3-dioxygenase (IDO) [25, 26]. After addition of 1MT to block IDO we found significant abrogation of the immunosuppressive effects of L-MSCs, but not BM-MSCs, on T-cell proliferation (Figure 6, $p<0.001$). No effect on cytotoxic degranulation was observed (data not shown). In conclusion, L-MSCs inhibit $CD4^+$ and $CD8^+$ T-cell degranulation partly by PD-L1/PD-1 interaction and inhibit T-cell proliferation partly by IDO.

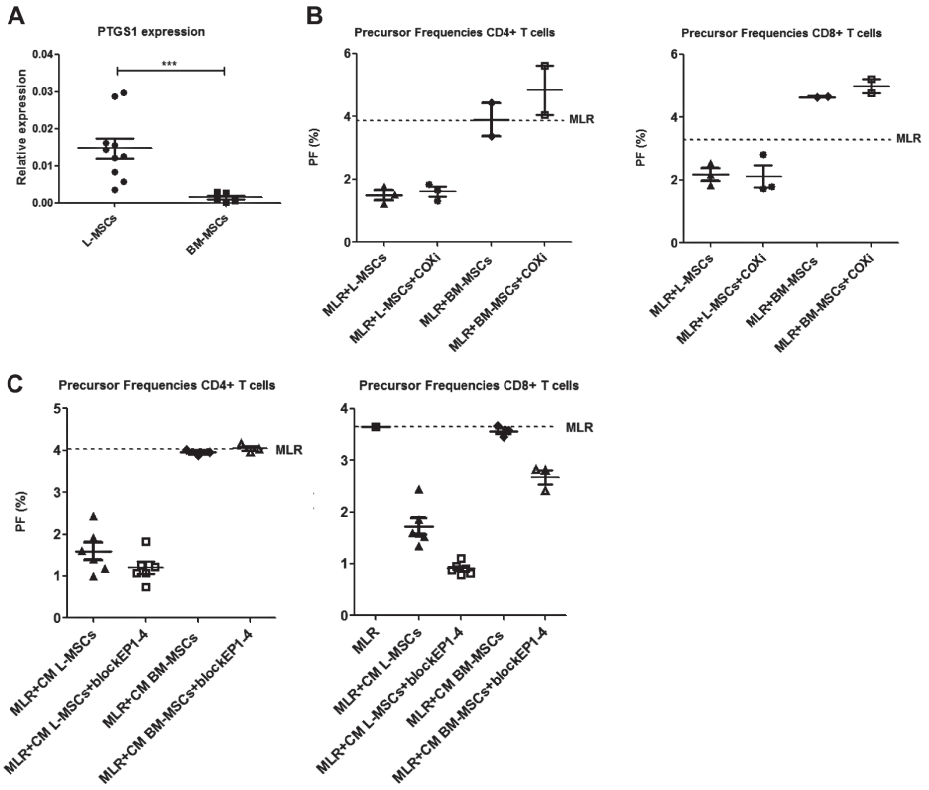

Figure 5.
Effects of blocking PGE_2 on the inhibition of T-cell responses by L-MSCs and BM-MSCs. (A) Relative expression of PTGS1 in L-MSCs (n=10) versus BM-MSCs (n=5). B) Scatter dot plots showing precursor frequencies of allo-reactive $CD4^+$ and $CD8^+$ T cells in the MLR without MSCs (control MLR, dotted line) and in the presence and absence of L-MSCs (n=3) versus BM-MSCs (n=2) pre-treated with and in the presence of COX-1 inhibitor Indomethacin. Data depicted as mean ± SEM. C) Scatter dot plots showing precursor frequencies of allo-reactive $CD4^+$ and $CD8^+$ T cells in MLRs with CM from L-MSCs (n=6) versus CM from BM-MSCs (n=3), in the presence and absence of blockers of PGE_2 receptors EP1-4. Data depicted as mean ± SEM. * $p<0.05$ ** $p<0.01$ *** $p<0.001$

DISCUSSION

In this study we showed that graft-derived L-MSCs which are released during transplantation suppress proliferation, cytotoxic degranulation and IFN-γ production of allo-reactive T cells. This suppressive capacity of L-MSCs was significantly better than that of BM-MSCs, even though the phenotype and differentiation potential of L-MSCs and BM-MSCs were very similar. However, L-MSCs expressed higher levels of PD-L1 than BM-MSCs. PD-L1 is the ligand for the co-inhibitory receptor PD-1 on T cells. On L-MSCs, expression of PD-L1 was associated with inhibition of T-cell proliferation and cytotoxic

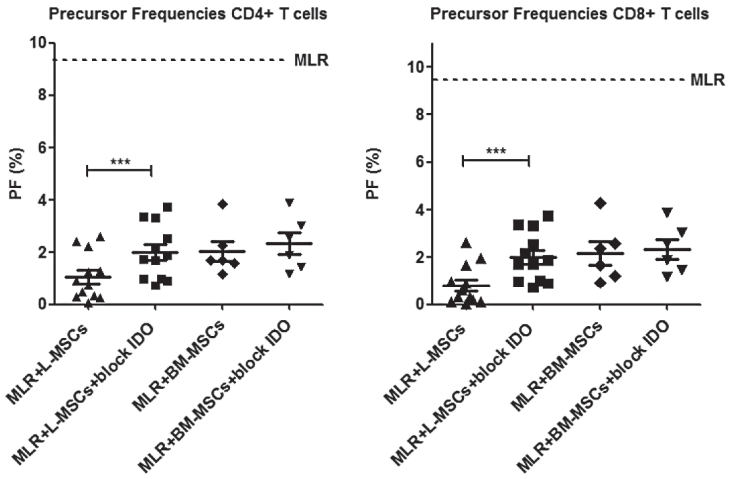

Figure 6.
Effects of blocking IDO on the inhibition of T-cell responses by L-MSCs and BM-MSCs. Scatter dot plots showing precursor frequencies of allo-reactive $CD4^+$ and $CD8^+$ T cells in the MLR without MSCs (control MLR, dotted line) and in the presence of L-MSCs (n=12) versus BM-MSCs (n=6), in the presence and absence of IDO-blocker 1MT. Data depicted as mean ± SEM. Differences between MLRs with and without 1MT were tested using Wilcoxon signed rank test. * $p<0.05$ ** $p<0.01$ *** $p<0.001$

degranulation in vitro. There was a strong correlation between levels of PD-L1 expression on MSCs and corresponding proliferation and cytotoxic degranulation of T cells in the co-cultures. The up-regulation of PD-L1 on graft-derived MSCs may be a consequence of *in vivo* priming by pro-inflammatory cytokines, which are elevated during the donation process [33, 34]. Blocking of PD-L1 in vitro partly abrogated the suppressive effects of L-MSCs on $CD4^+$ and $CD8^+$ T-cell cytotoxic degranulation. However, blocking of PD-L1 did not abrogate the effects on proliferation and IFN-γ and TNF-α production. Apparently, the PD-L1 expression represents a surrogate marker of inhibition, but the higher PD-L1 expression on L-MSCs only partly explains that suppressive effects of L-MSCs on T-cell responses were superior to those of BM-MSCs. Likely other mechanisms are involved in this.

We showed that, besides cell-cell contact-dependent mechanisms, soluble factors produced by L-MSCs contribute to the superior suppression of allo-reactive T-cell responses as well. Since soluble factors have been described to contribute to the immunosuppressive properties of MSCs (35) we further investigated the suppressive function of secreted factors of MSCs present in conditioned culture media on allo-reactive T-cell responses. Our finding that CM from L-MSCs suppressed T-cell proliferation, but not T-cell degranulation and IFN-γ and TNF-α production, whereas CM from BM-MSCs exerted no suppressive effects, suggests that L-MSCs produce T-cell suppressive soluble factors

that BM-MSCs do not produce. These soluble factors released by graft-derived L-MSCs may directly affect T cells or indirectly via their effect on antigen presenting cells [12, 36]. Soluble factors secreted by L-MSCs could inhibit the stimulator CD40-B cells in the co-cultures, which thereby causes inhibition of the T-cell responses.

An interesting finding was that L-MSCs showed higher expression of PTGS1, a key enzyme involved in prostaglandin production, than BM-MSCs. Although prostaglandins are produced by MSCs and are able to inhibit T-cell responses (9), we did not find abrogative effects of blocking prostaglandins, either by Indomethacin or by prostaglandin receptor inhibitors. Clearly these results show no role for PGE2 in the inhibition by MSCs.

More importantly, we found that blocking IDO partly restored T-cell proliferation that was inhibited by L-MSCs, while this had no effect on the conditions with BM-MSCs. IDO has previously been shown to inhibit T-cell responses by MSCs [25, 26, 37]. Our finding that blocking IDO only abrogated the inhibition by L-MSCs and not BM-MSCs supports the hypothesis that the immunomodulatory function of MSCs is highly plastic, as published previously (38), since L-MSCs have been exposed to an inflammatory environment.

One of the limitations of comparative studies of MSC populations is the lack of consensus to immunophenotypically define these cells (6). The current study compared MSC populations from liver grafts and bone marrow. MSCs found in liver graft perfusates adhered to plastic, were amenable to expansion and expressed cell surface markers that are described to identify MSCs, similar to BM-MSCs (6). The superior suppressive capacity of L-MSCs may be related to the tissue source. The phenotype of MSCs in the liver may reflect the hypo-responsiveness of the hepatic immune system. Another difference is that L-MSCs have been exposed to extensive ischemia, whereas cells from bone marrow were not exposed to substantial ischemia. Lastly, a difference between BM-MSCs and L-MSCs is their mobilization. L-MSCs were mobilized and released from the liver parenchyma during the donation process, whereas BM-MSCs were obtained from the stroma, without mobilization and release. Which of these differences is most likely to attribute to the T-cell suppressive capacity of MSCs needs further evaluation.

For future clinical applications, our study provides insights in the superior immunosuppressive capacity of L-MSCs, which may lead to cell-based immunosuppressive therapies using liver graft-derived MSCs. For this therapeutic application, L-MSCs need to be isolated from the donor liver and expanded in vitro, as described previously (39), since L-MSCs are present in low numbers. After expansion, L-MSCs should be infused in patients and may have an important function in the setting after liver transplantation (39) or may be used as bridging therapy before transplantation in patients with acute liver failure [40, 41].

In conclusion, we showed that MSCs mobilized from livers grafts strongly suppress allo-reactive T-cell responses. This suppression was stronger than non-mobilized and non-primed MSCs derived from BM stroma. These differences may not only be related

to the intragraft immune priming of L-MSCs in the organ donor or due to mobilization of MSCs from the graft, but may also be related to the inherent immunological tolerogenicity of the liver. In the setting of liver transplantation, resident and/or mobilized L-MSCs may therefore regulate immune-mediated liver damage by suppressing pathogenic alloreactive T-cell responses, leading to better acceptance of liver grafts.

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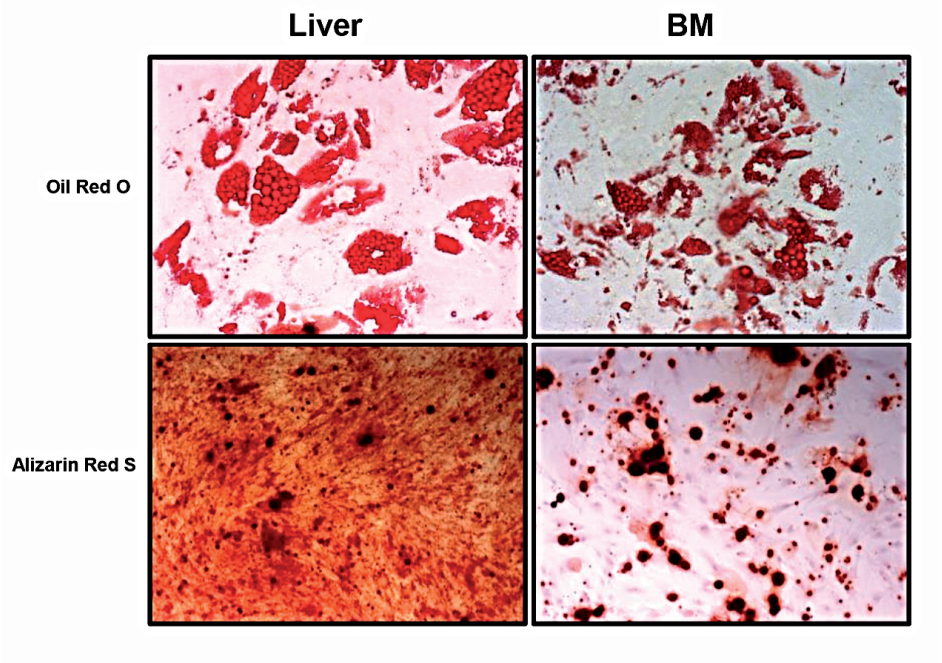

Supplemental Figure 1.
Differentiation potential of the plastic adherent MSC-like cells recovered from liver perfusates and from BM. Representative image of adipogenic differentiation depicted by uptake of Oil Red O (upper panels). Osteogenic differentiation potential depicted by Alizarin Red S staining (lower panels).

PART IV

Summary and discussion

CHAPTER 9

Summary and discussion

Liver transplant patients are susceptible to severe complications that strongly affect their survival and quality of life (1). The aims of this thesis were to identify risk factors for the occurrence of complications in patients with end-stage liver disease (ESLD), both before and after liver transplantation (LTx), and to develop strategies to optimize immunosuppression after LTx in order to reduce immunosuppression-related complications. Important complications in LTx patients are acute rejection and side effects of immunosuppressive drugs, such as infections. For example, up to 80% of LTx patients develop at least one episode of infection during the first year after transplantation, which is the leading cause of death early post-LTx (2, 3), and about 30% of LTx patients experience one or more episodes of acute rejection. Other factors that influence the occurrence and severity of these complications can be subdivided into different categories, amongst which genetic factors and viral factors. How these factors may affect complications in LTx patients, and which strategies may be used to reduce complications of the use of immunosuppression after LTx has been investigated in this thesis and our findings will be summarized and discussed in this chapter. We will also discuss which issues need to be addressed in future studies.

I. Genetic factors affecting susceptibility to infections and rejection in liver transplant patients

Summary

In part I (**Chapters 2-4)** of this thesis we focused on genetic factors in innate immunity receptors and their downstream inflammatory response genes that may affect susceptibility to infections in patients before and after LTx and to acute rejection after LTx.

In **Chapter 2** we investigated the allelic variants in the gene encoding the pro-inflammatory cytokine Tumor Necrosis Factor (TNF)-α. We showed that presence of the variant allele TNFA c.238A (rs361525) in ESLD patients almost completely protects from developing severe bacterial infections (SBIs) and SBI-related death in two independent cohorts of patients awaiting LTx. In addition, the A allele was associated with lower serum levels of TNF-α than the GG genotype. Although one could speculate that lower levels of the pro-inflammatory cytokine TNF-α may be associated with higher risk of infections, the strong protective effect of this allele supports the biological concept that TNF-mediated desensitization of innate immune cells drives increased susceptibility to bacterial infections in patients with ESLD (4-6). TNFA rs361525 genotyping in patients waiting for LTx may help to determine the individual risk of SBIs, which is helpful to decide on the use of prophylactic antibiotics in these patients.

In **Chapter 3** we studied the predictive value of donor mannose-binding lectin (MBL) genotyping for bacterial infections in patients after LTx in our own center, since three previous studies showed that patients transplanted with genetically MBL-insufficient

donor livers experienced more bacterial infections after LTx (7-9), while a fourth study found no association between donor MBL genotype and post-transplant infections (10). We noted that each of these studies categorized the donor livers differently with regard to MBL-sufficiency. We therefore categorized the donor livers in our study similar to the stratification systems that were used in the cited studies. Importantly, none of the three stratifications resulted in a statistically significant association between the donor MBL genotype and clinically significant infections in our patient cohort.

In **Chapter 4** we studied associations between genetic variants in various innate immunity receptors and the risk of bacterial and fungal infections as well as acute rejection in two cohorts of patients after LTx. Our data show that neither risk of bacterial and fungal infections nor risk of acute rejection after LTx is significantly influenced by 50 common genetic polymorphisms in innate immunity receptors. Since some of the investigated genetic variants in innate immunity receptors were found to be associated with risk of infections in critically ill non-transplanted patients (11-19), our findings suggest that the influence of these genetic variants is less prominent in transplanted patients. We believe that transplant-related variables therefore are superior to the tested genetic polymorphisms as risk factors for bacterial and fungal infections and acute rejection.

Discussion and future perspectives

Since up to 80% of LTx patients develop at least one episode of infection during the first year after transplantation (2, 3), and about 40% of patients waitlisted for LTx suffer from severe infections (**Chapter 2**), it is important to identify risk factors for infections in these patients. In this part we studied whether genetic variants in the innate system and their downstream inflammatory response genes affect the risk of infections in LTx patients, since innate immunity receptors trigger innate immune responses against microbial components and are also critically involved in shaping adaptive anti-microbial immune responses (11, 20, 21), and it has been described that genetic variants in these receptors are associated with the risk of infections in critically ill non-transplantation patients (11-19). In addition, since immunosuppressive drugs suppress the adaptive immune system of patients after LTx, the role of the innate immune system may become more important in combating infections. Although we also investigated associations between genetic variants and acute rejection and fungal infections after LTx, in this discussion we will focus on the effect of genetic variants on the risk of bacterial infections pre-LTx and post-LTx.

Interestingly, we found that a genetic variant in a cytokine gene of the Toll-like receptor (TLR) 4 signaling pathway, namely TNFA c.238A encoding the pro-inflammatory cytokine TNF-α, was significantly associated with the risk of bacterial infections in patients *before LTx* (**Chapter 2**), while in patients *after LTx* none of the studied genetic variants in innate

immune receptors were associated with bacterial infections (**Chapter 3-4**), although the TNFAc.238A polymorphism that we tested pre-LTx was not examined in our cohorts post-LTx. An important question that arises is: why did we not find associations between the genetic variants and infections after LTx while the study described in **Chapter 2** as well as other studies showed significant associations with infections in both immunocompetent and immunocompromised patients?

First, we showed in **Chapter 3** that previous studies that found an association between donor MBL2 variants and infections post-LTx used different ways to stratify donor livers into MBL-sufficient and MBL-insufficient on basis of genetic MBL polymorphisms (7-9). We have concerns about the validity of the results of these studies, because it is arbitrary which stratification method should be used, and the associations may have become insignificant when other stratification methods would have been used. However, none of the stratification methods resulted in significant associations between donor MBL2 variants and infections post-LTx in our study. This may be related to the time of follow-up, which was shorter in our study than in the other studies. This issue will be discussed below in more detail.

Second, in **Chapter 4** we showed that genetic variants in various innate immunity receptors in recipients as well in donors were not associated with the risk of infections after LTx, while in other studies significant associations were shown between some of these genetic variants and infections in critically ill non-LTx patients. These studies showed that genetic variants in TLR2, TLR4 and CD14, which were also studied in our study, were significantly associated with infections, specifically bacteremia and sepsis. TLR2 recognizes microbial membrane constituents such as lipoteichoic acid, peptidoglycan, and lipoproteins of gram-positive bacteria; and variants in this receptor may therefore mainly affect the risk of gram-positive infections (17). TLR4 binds gram-negative bacterial lipopolysaccharide (LPS). First, LPS is bound by circulating LPS-binding protein (LBP), which functions as an opsonin for CD14, which in turn acts as a catalyst for the binding of LPS to MD2, a co-receptor that is physically associated with TLR4. Genetic variants in TLR4, CD14 and/or LBP may therefore mainly affect the risk of gram-negative infections (13-16). For this reason, we analyzed associations between genetic variants and gram-positive or gram-negative infections also separately. Nevertheless, in contrast to the cited studies (11-19), we did not find any association between genetic variants in these receptors and clinically serious infections (CSI) in patients after LTx. A clinical factor that influences the risk of gram-negative or gram-positive infections is the use of selective bowel/digestive decontamination (SDD). Although SDD does not significantly increase the risk of CSI *in general*, it may cause a decrease in gram-negative bacterial infections, with an increased risk for gram-positive infections (22). In **Chapter 4** we described that patients from our study transplanted before the year 2000 received SDD, while patients transplanted after 2000 did not. In our study, patients with and without

SDD were not analyzed separately because the groups became too small; and thereby associations between variants in the TLR4 pathway and gram-negative infections and between TLR2 variants and gram-positive infections may have been missed. In addition, the lack of confirmation of associations between these variants and either gram-positive or gram-negative infections in our validation cohort may be explained by differences in SDD treatment as well, since patients in the validation cohort were transplanted in later years than patients in the identification cohort.

In addition, an important difference between critically ill non-transplant patients and post-LTx patients is that post-LTx patients use immunosuppressive drugs. As described in **Chapter 4**, not much is known about the influence of type and intensity of immunosuppression on expression of pattern recognition receptors (PRRs), although it has been shown that high dose steroid pulse therapy to treat acute rejection after LTx reduced expression of TLR2 and TLR4 on circulating monocytes (23). If immunosuppressive drugs affect the expression of PRRs in patients after LTx, this may explain that genetic variants in these receptors become less important in patients after LTx than in the general population.

Not many previous studies have investigated associations between genetic variants in receptors of the innate immune system and CSI in patients after LTx. Lee et al (24) showed that a polymorphism in TLR2 affected the *recurrence rate* of gram-positive infections and gram-positive septic shock in patients after LTx, but not the *incidence* of gram-positive infections. De Rooij et al showed that mutation of donor ficolin was associated with CSI and that the wild-type allele of donor mannan-binding lectin serine peptidase (MASP)2 was associated with CSI in two independent cohorts (8). A polymorphism in nucleotide-binding oligomerization domain (NOD)2 was associated with CSI in another study from the same research group (25). Interestingly, we did not find these associations in our cohorts, although the same polymorphisms were studied. An explanation for this difference may be that the studies used other follow-up times. We considered CSI only in the *first three months* after LTx, while the authors who found associations between variants in the lectin pathway and CSI (8, 25) studied infections within the *first year* after LTx. In Figure 1 of the study of de Rooij et al (8) it is shown that variants in different components of the lectin pathway have a cumulative effect on the risk of CSI, and this risk increases over time after LTx. Importantly, 3 months after LTx the difference in infection rate between patients with wild-type versus one variant in the lectin pathway is relatively small, but increases one year after LTx. In our study (**Chapter 4**) we may therefore have missed a significant influence of the genetic variants due to a shorter follow-up time and due to looking at the variants independently of each other, instead of combining them in order to find a cumulative effect.

In addition, it is known that infections at different time periods after LTx are influenced by different risk factors. As reviewed by Van Hoek et al (26), infections in the first month post-LTx are largely influenced by surgical risk factors and other transplant-related factors, while infections between one and six months post-LTx are more related to immunosuppression; and late infections (after six months post-LTx) may be influenced by co-morbidities such as hepatitis C, diabetes, and graft dysfunction. Due to the shorter follow-up time in our study, the influence of transplant-related factors on the incidence of CSI may be more prominent than in the other cited studies, and due to this, effects of genetic variants may have been missed in our study. In addition, although we included clinical variables in our multivariate analyses, unfortunately we did not include some of the risk factors that are known to contribute to CSI after LTx (26), such as prolonged ICU-stay, amount of intra-operative blood transfusion, and MELD-scores.

To further establish whether genetic variants in innate immunity receptors affect the risk of CSI after LTx, we suggest first to take into account the timing of infections, thereby studying infections in the first month post-LTx separately from infections between one and six months post-LTx. The effect of genetic variants in the innate immune system may be the strongest in the second time-frame, since this is the time-frame in which infections are most strongly affected by immunosuppression and less by surgical and transplant factors. Second, we suggest to include important transplant-related factors in the analyses, as mentioned above. Third, larger patient cohorts are needed, in order to enable stratification of patients into groups with different prophylactic antibiotic therapies, which also has been shown to influence the risk of CSI (26), and with and without SDD, which may influence the incidence of post-transplant gram-positive versus gram-negative infections, thereby affecting the relative influences of genetic variants in TLR2 and TLR4 pathways.

Importantly, the TNFA polymorphism that we showed to protect patients against infections pre-LTx (**Chapter 2**) was not determined in our patients post-LTx (**Chapter 4**). For future study, it would be very interesting to follow-up those of the patients studied in Chapter 2 who were transplanted, in order to determine whether the TNFA c.238 A allele also protects patients against infections and infection-related death after LTx. In addition, it is also interesting to retrospectively investigate whether the polymorphisms that we studied in Chapter 4 are associated with infections in these patients before they were transplanted.

For clinical practice, although we did not find significant effects of the other genetic variants in the TLR4 pathway that we tested in Chapter 2, it is interesting to investigate whether different combinations of genetic variants in this pathway or in other pathways are associated with the risk of infections in LTx patients, in order to establish a “genetic

risk profile" either with or without combination with clinical risk factors. This will increase the predictive value of these risk factors and contribute to identifying different patient categories who need less or more prophylactic antibiotic treatment and microbial monitoring.

II. Viral factors affecting anti-donor T-cell reactivity after liver transplantation

Summary

In Part II (**Chapter 5-6**) we described the influence of viral factors, especially cytomegalovirus (CMV) infection, on the T-cell alloresponse after LTx.

In **Chapter 5** we showed that patients with CMV primary infection after LTx show *ex vivo* donor-specific $CD8^+$ T-cell hyporesponsiveness. Moreover, in a large retrospective cohort study of LTx patients from our center we showed that patients with primary CMV infections are protected from the occurrence of late acute rejection (LAR). In addition, we determined circulating Vδ1 and Vδ2 γδ T-cell subsets and calculated the ratios between these two subsets. We found that CMV primary infection patients had higher Vδ1/Vδ2 T-cell ratios in peripheral blood than patients with other CMV serostatus. Higher Vδ1/Vδ2 T-cell ratios have been associated with operational tolerance in previous studies (27-29). We therefore conclude that CMV primary infection may promote tolerance towards the allograft in LTx patients.

In **Chapter 6** we investigated whether allogeneic T-cell responses after LTx are regulated by co-inhibitory receptors. It is known that persistent viral infections can induce the expression of these receptors on T cells, thereby enabling suppression by co-inhibitory ligands expressed in infected tissues (30-36). We hypothesized that persistent presence of alloantigens after LTx might induce co-inhibitory receptor expression on T cells and thereby suppression of donor-specific T-cell responses. Although experimental animal studies have shown involvement of co-inhibitory receptor-ligand interactions in liver allograft tolerance, the role of these receptors in T-cell alloresponses after LTx in humans has not been established yet. We showed that, already six months after LTx, expression of the co-inhibitory receptors CD160 and CD244 on circulating $CD8^+$ T cells of LTx patients was higher than before LTx, and the elevated expression was sustained late after LTx, with CD244 showing the most prominent increase. Interestingly, significant upregulation of CD244 on circulating $CD8^+$ T cells was restricted to patients who experienced CMV infection after LTx. CMV infection was also associated with reduced *ex vivo* $CD8^+$ T-cell proliferation and cytotoxic degranulation in response to allo-antigens late after LTx. Purified $CD244^+$ $CD8^+$ T cells of LTx patients showed lower proliferative responses than their CD244- counterparts to alloantigens as well as in response to polyclonal stimuli. In addition, the $CD244^+$ $CD8^+$ T-cell population contained the majority of CMV-MHC class I tetramer positive cells. In conclusion, CMV infection after LTx, rather than persistence of

alloantigen, is associated with the expansion of a dysfunctional $CD8^+CD244^+$ T-cell subset and development of systemic allogeneic $CD8^+$ T-cell hyporesponsiveness. These results suggest that CMV infection restrains $CD8^+$ T-cell alloresponses after LTx.

Discussion and future perspectives

As discussed in **Chapter 1**, T-cell responses to viruses, including CMV, have been proposed as one of the main barriers to achieve transplant tolerance (37), as shown in experimental animal models (38-40). As reviewed by D'Orsogna et al (41), this is probably caused by virus-specific memory T cells that cross-react against allogeneic HLA, resulting in increased T-cell responses against allogeneic organ grafts. In contrast to this, in both **Chapter 5** and **Chapter 6** we showed that CMV infection after LTx was associated with *ex vivo* allogeneic hyporesponsiveness of circulating $CD8^+$ T cells, which possibly favors tolerance towards the liver graft rather than rejection. Although there were some differences between the two studies, which will be discussed below, each of these studies has its own strengths. **Chapter 5** showed, besides the association between primary CMV infection and *ex vivo* allogeneic $CD8^+$ T-cell hyporesponsiveness, a clinical confirmation of this association, namely a significantly reduced incidence of late acute rejection episodes in LTx patients with primary CMV infection. In addition, we found an association between primary CMV infection and an increased ratio of circulating Vδ1/Vδ2 T cells, a marker that was found to be associated with operational tolerance after LTx in previous studies. **Chapter 6** provides a more mechanistic view on the association between CMV infection and T-cell hyporesponsiveness after LTx. This chapter adds to the study presented in Chapter 5 as it shows that CMV infection induces sustained expression of the co-inhibitory receptor CD244 on circulating $CD8^+$ T cells of patients after LTx. These $CD244^+$ T cells showed generalized hyporesponsiveness, both towards allo-antigens and a polyclonal stimulus, and this population contained the majority of CMV-specific $CD8^+$ T cells in CMV-infected LTx patients. These data suggested that CD244-induction is one of the mechanisms by which CMV infection induces $CD8^+$ T-cell hyporesponsiveness. However, we showed that blocking CD244 interaction with its ligand CD48 did not enhance responsiveness of $CD244^+$ $CD8^+$ T-cells to alloantigens. We therefore conclude that CD244 expression on $CD8^+$ T cells in CMV-infected LTx patients is rather a surrogate marker for the expansion of differentiated T cells with limited proliferative potential, than a mechanism that leads to hyporesponsiveness or tolerance.

There are several differences between these two chapters. First, in **Chapter 5** we determined circulating T-cell responses in patients early after LTx, while in **Chapter 6** patients late after LTx were studied. It is therefore not allowed to compare the results of the two studies one-to-one.

A second difference is that we used different assays to determine alloreactive CD8$^+$ T-cell responses: in **Chapter 5** we determined expression of the activation marker CD137 after 24 hours of allogeneic stimulation, while in **Chapter 6** CD8$^+$ T-cell proliferation and cytotoxic degranulation were measured after 5 days of allogeneic stimulation. Interestingly, we found in **Chapter 5** that early *activation* of alloreactive CD8$^+$ T cells was reduced donor-specifically in CMV-infected patients, and **Chapter 6** added to this that *proliferation* of alloreactive CD8$^+$ T cells was donor-specifically reduced as well in CMV-infected patients. In addition, **Chapter 6** showed impaired cytotoxic degranulation of alloreactive CD8$^+$ T cells in these patients, which was however observed for the response to both donor and third party allo-antigens. Apparently, in CMV-infected patients late after LTx CD8$^+$ T cells become generally exhausted and not only in response to donor alloantigens. The reason for this is unknown. It would be interesting to investigate this by performing a microarray experiment on CD244$^+$ versus CD244- CD8$^+$ T cells, since we showed that CD244$^+$ CD8$^+$ T cells are induced in CMV-infected patients, and are hyporesponsive in response to stimulation with both donor and third party allo-antigens. In addition, this experiment should be performed for patients early and late after LTx, to determine whether early after LTx these cells are donor-specifically hyporesponsive while they may become generally hyporesponsive late after LTx.

A third difference between the two studies is that in **Chapter 5** the association between CMV infection and CD8$^+$ T-cell hyporesponsiveness was only observed in CMV primary infection patients, but not CMV reactivation patients (therefore reactivation patients were taken as one category together with IgG$^+$ CMV non-reactivation patients), while in **Chapter 6** the association was found for both primary infected patients and reactivation patients (therefore primary infection and reactivation patients were combined into one category: CMV infection patients). An explanation for this difference may be related to the method of categorizing patients into four groups of CMV status: 1) CMV naïve (CMV IgG- recipient transplanted with a liver from a CMV IgG- donor, (R-/D-)); 2) CMV primary infections (CMV IgG- recipient who gets a first CMV infection after LTx due to transplantation of a liver from a CMV IgG$^+$ donor (R-/D+), and infection is determined either by positive PCR > 50 DNA copies/mL or CMV IgG seroconversion; 3) CMV non-reactivations (CMV IgG$^+$ recipient who does not experience CMV reactivation after LTx); and 4) CMV reactivations (CMV IgG$^+$ recipient who does experience CMV reactivation after LTx, either determined by positive PCR > 50 DNA copies/mL or positive CMV IgM). Distinction between patients in category 3 and 4 is however often difficult, because no protocolled CMV monitoring is performed in patients falling into these categories and reactivation could be easily missed when diagnostics are not performed, for example because clinical signs of CMV infection are missing or misinterpreted. This may lead to underestimation of the number of patients in category 4 (which are more hyporesponsive), together with

overestimation of the number of patients in category 3. As a consequence of this, the difference in $CD8^+$ T-cell responsiveness between the two categories may be underestimated, since there have probably patients been included in category 3 having $CD8^+$ T-cell hyporesponsiveness that actually belong to category 4. It may therefore be a wise solution to combine patients in categories 3 and 4, which was done in **Chapter 5**. However, in **Chapter 6** we observed large differences in $CD8^+$ T-cell responses between the two categories, so even if the difference was underestimated the results will remain similar. However, to gain more insights into allogeneic T-cell responses in categories 3 and 4, a future study with patients in which CMV PCRs are performed by protocol in all patients will be needed.

Another interesting subject to investigate in future studies is the link between CMV infection and operational tolerance after LTx. Our findings, showing that CMV infection is associated with $CD8^+$ T-cell hyporesponsiveness, induction of CD244 expression, fewer LAR, and higher Vδ1/Vδ2 T-cell ratios, lead to the hypothesis that CMV infection may result in operational tolerance in LTx patients. To investigate such association, a prospective trial in which immunosuppressive drugs are weaned off in LTx patients, combined with determination of $CD8^+$ T-cell responses and CMV status, is required. If this association would be proven to exist, CMV status may easily be taken into account by physicians when selecting LTx patients for tapering or withdrawing immunosuppressive therapy, since it is easily measured without additional effects or costs. In addition, our current studies do not provide clues for a causal relationship between CMV infection and reduced systemic $CD8^+$ T-cell alloresponsivity. In **Chapter 6** we discussed that the T-cell hyporesponsiveness that we observed in CMV-infected patients may be related to several mechanisms, such as: limited T-cell receptor (TCR) repertoire of CMV-specific T cells; T-cell senescence and shorter telomere length of CMV-specific T cells, resulting in limited proliferative capacity; IFN-α production due to chronic inflammation caused by CMV, resulting in impaired memory T-cell responses; and finally production of viral IL-10 by CMV. Future studies are needed to investigate which of these mechanisms cause the $CD8^+$ T-cell hyporesponsiveness in CMV-infected patients after LTx. First, we suggest to perform immunophenotypic analysis of the TCR-Vbeta repertoire in LTx patients with different CMV serostatus, combined with determination of $CD8^+$ T-cell alloresponses, to test the hypothesis that CMV-infected patients have a limited TCR repertoire, resulting in T-cell hyporesponsiveness. Second, telomere length of $CD8^+$ T cells should be measured in LTx patients with and without CMV infection. Third, we suggest to determine IFN-α and IL-10 mRNA expression, preferentially in liver grafts from CMV-infected versus non-infected patients. A good source to study this may be liver biopsies from both CMV-experienced and non-experienced LTx patients to compare both groups.

III. Optimization of immunosuppression after liver transplantation

Summary

After LTx, all patients are treated with immunosuppressive drugs to prevent rejection of the liver graft. In **Chapter 7** of this thesis we described that from the 1980s calcineurin inhibitors (CNIs), such as cyclosporine A (CsA) and tacrolimus (TAC), have been the most used immunosuppressive drugs after LTx. These drugs inhibit calcineurin, which via an intracellular signaling cascade leads to inhibition of a family of transcription factors, resulting in reduced transcriptional activation of early cytokine genes, such as IL-2, TNF-α, IL-3, IL-4, granulocyte-macrophage colony-stimulating factor, and IFN-γ (42). Ultimately, proliferation of T cells and production of pro-inflammatory cytokines by T cells are reduced. Due to inhibition of T-cell reactivity, allograft rejection is prevented. However, due to the generalized inhibition of T-cell reactivity, which is also needed to combat pathogens, the use of CNIs involves a serious risk of infections. Other side effects of CNIs are nephrotoxicity, neurotoxicity, hypertension, *de novo* malignancy and new-onset diabetes mellitus. Since studies from the 1990s onwards showed that TAC was associated with less nephrotoxicity and neurotoxicity than CsA, TAC has become the first choice maintenance immunosuppressant after LTx, often combined with prednisolone (43). However, as described in **Chapter 7**, because of the side effects that still may occur due to the use of TAC, several strategies have been developed to minimize the dosage of TAC. We described four strategies:

1. *Switch to CNI-free immunosuppressive regimen when CNI-toxicity has evolved.*
2. *Start with mTOR inhibitor in combination with other immunosuppressive drugs directly post-LTx (CNI-free regimen)*
3. *Delayed introduction of reduced-dose tacrolimus under protection of mycophenolate mofetil (MMF) and dacluzimab*
4. *Switch to CNI-free regimen early after LTx*

In **Chapter 8** we hypothesized that the immunosuppressive properties of liver graft-derived MSCs (L-MSCs) are superior to those of bone marrow-derived MSCs (BM-MSCs). We investigated the immunosuppressive effects of MSCs from both sources on allogeneic T-cell responses. For this purpose, we isolated MSCs from liver graft perfusates. In this chapter we described that proliferation, cytotoxic degranulation and IFN-γ production of allo-reactive T cells were more potently suppressed by L-MSCs than by BM-MSCs. Suppression was mediated both by cell-cell contact and secreted factors. The latter was established by the finding that in cultures with conditioned media (culture medium derived from MSC cultures) (CM) from L-MSCs also suppressed T-cell responses, while CM from BM-MSCs were not suppressive. In addition, L-MSCs showed *ex vivo* a higher

expression of PD-L1 than BM-MSCs, which was associated with more potent inhibition of T-cell proliferation and cytotoxic degranulation *in vitro*. Blocking PD-L1 partly abrogated the inhibition of cytotoxic degranulation by L-MSCs. In addition, blocking IDO partly abrogated the inhibitory effects of L-MSCs, but not BM-MSCs, on T-cell proliferation. In conclusion, liver graft-derived MSCs suppress allogeneic T-cell responses stronger than BM-MSCs, which may be related to *in situ* priming and mobilization from the graft. These graft-derived MSCs may therefore be relevant in liver transplantation by suppressing recipient T-cell responses to the allogeneic liver graft.

Discussion and future perspectives

In **Chapter 7** we stated that calcineurin inhibitors (CNIs) are the cornerstone of maintenance immunosuppression after LTx, despite their adverse effects. To minimize these adverse effects, we propose to optimize the use of CNIs by developing new treatment strategies that minimize or even replace the use of CNIs after LTx. Whether the use of mammalian target of rapamycin (mTOR) inhibitors will reduce the incidence of malignancy after liver transplantation needs to be studied in the future. Regarding kidney function after LTx, a recent 24-month prospective, randomized, multicenter, open-label study with *de novo* liver transplant patients was performed to compare kidney functions in patients treated with tacrolimus (TAC) versus the combination of everolimus (EVR) and reduced TAC (44, 45). In this study, patients were randomized at 30 days to everolimus (EVR) combined with reduced tacrolimus (TAC; n = 245), TAC Control (n = 243) or TAC Elimination (n = 231). The last group was prematurely terminated because of high rates of acute rejections. This study showed a significantly lower incidence of acute rejection episodes in the combination group than in the TAC control group, and, more importantly, a significantly better kidney function at 2 years after LTx in patients treated with combination therapy than with TAC alone. Another study is ongoing in our center, in which standard dose TAC is compared with a combination of reduced dose sirolimus and reduced dose TAC to study the effects on kidney function, malignancy and adverse effects such as *de novo* diabetes mellitus and hypertension. Results of this study are awaited in the coming years. Because of the adverse effects of TAC, CNI minimization strategies need further exploration to improve long-term outcome after liver transplantation.

Besides studies to develop clinical CNI minimization strategies, basic research should focus on finding alternative treatment strategies in order to replace or minimize immunosuppressive drugs that are used nowadays. For that purpose, as mentioned in **Chapter 1**, cell-based treatment strategies are under investigation, of which MSC-therapy is of special interest because of the immunosuppressive capacities of these cells. In previous studies, it has been shown that MSCs may have the following immunosuppressive properties: 1) inhibition of T-cell proliferation (46-50); 2) induction of regulatory T cells

(Tregs) (51); 3) inhibition of T-cell stimulatory capacity of DCs (52); and 4) differentiation of macrophages towards a regulatory phenotype and function (53). MSCs can be isolated from various tissues, such as bone marrow, adipose tissue, and many organs including liver (54-57). The cited studies showed that MSCs from all these locations have immunosuppressive properties, as these studies used MSCs isolated from various tissues and anatomical compartments of the human body. In a previous study from our research group, it was shown that L-MSCs are immunosuppressive as well (57). In **Chapter 8** we compared for the first time the immunosuppressive effects of L-MSCs and BM-MSCs on allogeneic T-cell responses. Although MSCs from both sources suppressed T-cell responses strongly *in vitro*, we interestingly found that L-MSCs were significantly more suppressive than BM-MSCs. We also showed that PD-L1 and IDO were involved in suppression of T-cell responses by L-MSCs. We hypothesized that these differences are not only related to the intragraft immune priming of L-MSCs in the organ donor or due to mobilization of MSCs from the graft, but also to the inherent immunological tolerogenicity of the liver. Although future studies are needed to provide more insight into the mechanisms that render L-MSCs superior to BM-MSCs in suppressing allo-reactive T-cell responses, we would like to speculate further on the possible clinical application of L-MSCs as alternative immunosuppressive treatment in LTx patients.

Most of the evidence regarding safety and efficacy of MSCs as a therapeutic agent in solid organ transplantation comes from the Expert Meetings of the Mesenchymal Stem Cells in Solid Organ Transplantation (MiSOT) Consortium (58). These meetings focus on translation of preclinical data into early clinical settings in which MSC-therapy is used after kidney transplantation. To our knowledge, no clinical studies have been performed on MSC-therapy in LTx patients yet. In patients with liver disease however, studies have shown that administration of MSCs reduced liver fibrosis and was beneficial in patients with acute liver failure as bridging therapy before LTx (59, 60).

When envisioning application of MSC-therapy after solid organ transplantation, the following steps need to be performed. First, MSCs need to be isolated from bone marrow, adipose tissue or organ tissue. Then they need to be expanded *in vitro*, as previously described (61). After expansion, the cells are intravenously (i.v.) infused into the patient. Although a study in mice by Eggenhofer et al demonstrated that MSCs are short-lived after i.v. infusion and that viable MSCs do not pass the lungs (62), to date, this strategy of MSC-administration in humans has been shown to be relatively safe and feasible in kidney transplantation patients. Eggenhofer et al stated that long-term immunomodulatory and regenerative effects of infused MSC must therefore be mediated by other cell types. Tan et al showed that MSC-therapy prevented acute cellular rejection and reduced the need for induction and maintenance immunosuppression (63). In addition, a pilot study on safety and feasibility of MSC-therapy in two kidney transplant patients, performed

by Perico et al, showed that MSC-administration was safe, and both patients had long-term stable graft function (64), and another study showed reduction of tubulitis and interstitial fibrosis/tubular atrophy in some patients (65). In addition, MSC-therapy after kidney transplantation may induce systemic alloimmune modulation: a donor-specific down-regulation of the allogeneic proliferation of PBMCs was reported after autologous BM-MSC-therapy (65) and higher ratios of circulating regulatory T cells (Tregs) to memory T cells were reported in another study (64).

Several issues regarding MSC-therapy need to be considered. First, the optimal timing of MSC-infusion in transplant patients is still a matter of debate. The most recent MiSOT position paper (58) advocates that the timing of MSC-administration depends on the therapeutic goal and on concomitant immunosuppressive drugs: to induce a tolerogenic state and prevent early acute rejections, MSC may be given around transplantation (64), while later administration after transplantation may be needed for treatment of ongoing (subclinical) chronic rejection. However, long-term effects of MSCs on chronic rejection are still awaited (58).

A second issue of discussion is the use of concomitant immunosuppressive drugs. In all safety trials regarding MSC-therapy in kidney transplant patients, patients used CNIs as maintenance immunosuppression. It was however shown in preclinical studies that mycophenolic acid may have a synergistic immunosuppressive effect together with MSCs (66), while CNIs have not (67). In future clinical studies, different combinations of immunosuppressive drugs together with MSCs need to be investigated. Another remaining question that needs to be addressed in future studies is whether MSCs may be used instead of induction immunosuppressive therapy with basiliximab, since previous studies showed conflicting results (63, 64).

Other important issues that have been investigated but still need attention in future studies are the potential oncogenicity of MSCs and the occurrence of opportunistic infections in patients undergoing MSC-therapy. Although there is currently no evidence that MSCs induce malignancies (68), other studies showed that MSCs during expansion before clinical application were at risk for genetic alterations (69, 70). Regarding the occurrence of infections, one study showed a higher risk of opportunistic infections in patients receiving MSC-therapy after kidney transplantation (65), while another study showed a protective effect of MSCs on the occurrence of infections (63).

The last issue regarding clinical applicability of MSC-therapy in solid organ transplantation we would like to discuss is the source of MSCs. Since **Chapter 8** focused on comparing immunosuppressive capacities of MSCs from two different sources, this topic is

very much related to our study. As described above, MSCs can be isolated from various types of tissues (54-57) and they may be of autologous or allogeneic origin. As reviewed by Roemeling-Van Rhijn et al (71), for clinical application, MSCs from allogeneic origin have the advantage that they can be isolated from bone marrow aspirates of healthy individuals, culture expanded and cryopreserved, after which they are readily available for infusion at any moment, for example immediately during transplantation. However, autologous MSCs may be less immunogenic than allogeneic MSCs, with lower risk of induction of an immune response against MSCs. This is however still a subject for future study, since previous studies on the one hand showed that allogeneic MSCs do not provoke immune responses (46, 72), while on the other hand other studies showed that allogeneic MSCs may be recognized by the adaptive immune system (73-75), which may result in rapid clearance of MSCs (76). In the clinical trials performed so far, only autologous MSCs have been used. Using autologous MSCs from patients awaiting LTx is another option to consider. However, isolating MSCs from bone marrow aspirates of patients on the waiting list for LTx may be debilitating for those patients who are in bad condition. Although the immunogenicity of allogeneic MSC needs further study, a very recent pilot study showed that administration of donor-derived BM-MSC was safe and allowed reduction of conventional dose of tacrolimus in living-related kidney transplant recipients, at least during 12 months of follow-up (77). Since we showed in **Chapter 8** that donor liver graft-derived MSCs suppressed allogeneic T-cell responses better than BM-MSCs (both of allogeneic origin), we think that perfusates from the donor liver may be a very good source of MSCs for immunosuppressive therapy after LTx. A disadvantage of this source is that MSCs are not available at time of transplantation, because they need to be expanded before infusion. However, it will be possible to administrate the cells a few weeks after LTx. In addition, it is questionable whether it is possible to expand L-MSCs to a sufficient number of cells in order to make them suitable for MSC-therapy. In the cited studies in which BM-MSCs showed to be immunosuppressive in kidney transplant patients 1-2*10^6 MSCs/kg were infused (63, 65), and according to the expansion data that we showed in **Chapter 8** (Figure1B), this number of cells may be achievable for some, but not all, donor-livers. Future studies in LTx patients are needed to determine whether sufficient immunosuppressive effects can be achieved by lower numbers of L-MSCs, since L-MSCs showed to be much more suppressive than BM-MSCs.

Overall conclusion

In this thesis we showed that genetic and viral factors importantly contribute to the risk of complications in liver transplant patients, although the impact of genetic factors showed to be more prominent before LTx than after LTx. In addition, we described clinical strategies to optimize the use of immunosuppressive drugs after LTx and showed basic findings that may lead to cell-based immunosuppressive treatment. In Table I the main

findings of this thesis are summarized, including strengths and limitations of our findings. In addition, based on the findings described in this thesis, a summary of suggestions for application of the findings in clinical practice ('clinical practice points'), as well as suggestions for future study are provided in this table.

Table I. Summary and discussion of the main findings of this thesis

Chapter	Main findings	Strength	Limitations	Clinical practice points	Suggestions for future study
2	TNFA c.238 A allele protects ESLD patients against SBIs and SBI-related death	- Two independent cohorts - Strong predictive value - Functional effect of SNP on TNF-α levels	No follow-up post-LTx	Less prophylactic antibiotics and monitoring in low risk patients	- Develop genetic risk profile - Follow-up patients post-LTx
3	Donor MBL2 genotype does not predict risk of infections post-LTx	- Large cohort - Three stratification strategies used	Short follow-up	Early after LTx clinical factors superior to genetic factors in conferring risk to infections	- Longer follow-up - Combine with other SNPs
4	Genetic variants in various innate immunity receptors do not predict risk of infections and rejection post-LTx	- Two independent cohorts - Clinical risk factors included	- Not all transplant-related risk factors included - Short follow-up and first month included in follow-up - No stratification based on clinical variables (limited sample size)	Early after LTx clinical factors superior to genetic factors in conferring risk to infections	- Study infections in different time-frames separately - Longer follow-up - Include more transplant-related risk factors - Larger cohort - Study effect of immunosuppressive drugs on PRRs
5	- CMV infection is associated with donor-specific $CD8^+$ T-cell hypo-responsiveness early post-LTx - CMV infection protects against LAR - CMV infection is associated with a marker for operational tolerance after LTx: higher Vδ1/Vδ2 ratio.	- In vitro data clinically confirmed in large cohort - Both in vitro and clinical data in line with marker for operational tolerance	- Only short-term culture assay used to determine $CD8^+$ alloresponses - No data on causality CMV infection and hypo-responsiveness/ tolerance	Consider CMV serostatus when tapering/ withdrawing immunosuppressive drugs in LTx patients	Set up prospective weaning trial to study association between CMV and tolerance

Table I. Summary and discussion of the main findings of this thesis (continued)

Chapter	Main findings	Strength	Limitations	Clinical practice points	Suggestions for future study
6	- CMV infection induces CD244 expression on $CD8^+$ T cells in patients early and late after LTx - CMV infection is associated with $CD8^+$ T-cell hypo-responsiveness late after LTx - Purified $CD244^+$ $CD8^+$ T cells showed lower proliferative responses than CD244- cells to allo-antigen - $CD244^+$ $CD8^+$ T-cell population contained the majority of CMV-tetramer positive cells	- Data on both early and late post-LTx - More mechanistic insights into effects of CMV on $CD8^+$ T-cell responses - First study investigating role of inhibitory receptors in allo-response after LTx	No data on causality CMV infection and hypo-responsiveness/ tolerance	CD244 possible biomarker for CMV infection post-LTx, which may identify tolerant patients	Set up prospective weaning trial to study association between CMV and tolerance
7	Review describing clinical CNI optimization strategies	Strategies to minimize CNI exposure will improve outcome after liver transplantation	Despite optimization of the use of CNIs, patients still suffer from side effects affecting survival and quality of life	- CNIs are still the cornerstone of immunosuppresion after LTx, with tacrolimus as first choice - CNIs have a narrow therapeutic window necessitating close drug monitoring - Conversion from the tacrolimus BID to tacrolimus QD formulation in stable LTx patients is safe and will increase adherence and quality of life	- CNI minimization strategies need further exploration to improve long term outcome after liver transplantation - basic research should focus on finding alternative treatment strategies

Table I. Summary and discussion of the main findings of this thesis (continued)

Chapter	Main findings	Strength	Limitations	Clinical practice points	Suggestions for future study
8	- L-MSCs suppress proliferation, cytotoxic degranulation and IFN-γ production of allo-reactive T cells better than BM-MSCs - Higher PD-L1 expression on L-MSCs than BM-MSCs, associated with stronger suppressive effects - Suppression of degranulation by L-MSCs partly mediated by PD-L1 - Suppression of proliferation by L-MSCs partly mediated by IDO	- First study comparing immunosuppressive effects of L-MSCs versus BM-MSCs - Effects of both cell-contact dependent and conditioned media tested - Functional effects of PD-L1, PGE2 and IDO tested	Unknown yet what the reason is that L-MSCs suppress T-cell responses better than BM-MSCs	No clinical practice points yet	- Assess whether differences in suppressive capacity of L-MSCs and BM-MSCs are related to source, exposure to ischemia, and/or release from liver parenchyma during donation process - Perform clinical phase I trial in liver transplant patients

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CHAPTER 10

Nederlandse samenvatting / Dutch summary

Levertransplantatiepatiënten zijn vatbaar voor ernstige complicaties die hun overleving en kwalieit van leven sterk beïnvloeden. De doelen van dit proefschrift waren: risicofactoren identificeren voor het optreden van complicaties in patiënten met eindstadium leverziekte, zowel voor als na levertransplantatie (LTx) en strategieën ontwikkelen om het gebruik van afweeronderdrukkende medicijnen (immunosuppressiva) te optimaliseren/verminderen. Dit laatste heeft als doel complicaties die gepaard gaan met het gebruik van deze medicijnen te voorkómen of verminderen. Belangrijke complicaties in LTx patiënten zijn acute afstoting van hun lever transplantaat en bijwerkingen van immunosuppressiva, zoals infecties. Ongeveer 80% van de LTx patiënten krijgt namelijk tenminste één infectie tijdens het eerste jaar na LTx en infectie is de meest voorkomende doodsoorzaak in patiënten vroeg na LTx. Daarnaast krijgt ongeveer 30% van de LTx patiënten één of meerdere keren te maken met afstoting van hun donor lever. Naast het gebruik van immunosuppressiva zijn er andere factoren die het optreden en de ernst van complicaties in LTx patiënten beïnvloeden en deze factoren zijn onder te verdelen in een aantal categorieën, waaronder *genetische* en *virale* factoren. Hoe deze factoren complicaties in LTx patiënten beïnvloeden en welke strategieën gebruikt kunnen worden om complicaties van immunosuppressiva te verminderen, is onderzocht in dit proefschrift. In dit hoofdstuk worden de belangrijkste resultaten samengevat en bediscussieerd. Verder zal hier worden besproken welke zaken er zouden moeten worden onderzocht in toekomstige studies.

I. Genetische factoren die vatbaarheid voor infecties en afstoting in levertransplantatiepatiënten beïnvloeden

Samenvatting

In Deel I (**Hoodstukken 2-4**) van dit proefschrift hebben we onderzocht of variaties in genen die coderen voor receptoren van het “innate immuunsysteem” (oftewel het aangeboren, niet-specifieke immuunsysteem) vatbaarheid voor infecties in patiënten voor en na LTx en voor afstoting na LTx kunnen beïnvloeden.

In **Hoofdstuk 2** hebben we ingezoomd op variaties in genen die coderen voor een cytokine (signaalstof) genaamd Tumor Necrosis Factor Alpha (TNF-α). In dit hoofdstuk hebben we gevonden dat de aanwezigheid van een variant allel, namelijk het A-allel, in het gen dat codeert voor TNF-α in patiënten met eindstadium leverziekte op de wachtlijst voor LTx zeer sterk beschermt tegen het optreden van ernstige bactierële infecties en tegen overlijden ten gevolge van een ernstige infectie. Verder vonden we lagere hoeveelheden TNF-α in het bloed van patiënten met het A-allel dan in patiënten met twee G-allelen. Deze bevindingen zijn in lijn met wat eerder is aangetoond: meer TNF-α leidt tot uitputting van immuuncellen, waardoor de vatbaarheid voor infecties groter wordt.

Het bepalen van deze genetische variatie in het TNF-α gen in patiënten op de wachtlijst voor LTx is daarom van nut om het risico op infecties per individu in te schatten, wat kan helpen in de keuze voor het al dan niet preventief geven van antibiotica.

In **Hoofdstuk 3** hebben we onderzocht of genetische variaties in genen die coderen voor mannose-bindend lectine (MBL), een eiwit van het innate immuunsysteem, van leverdonoren voorspellend zijn voor het optreden van infecties in patiënten na LTx. Op basis van deze genetische variaties zijn donorlevers in te delen in levers met *voldoende* of *onvoldoende* MBL. Drie eerdere onderzoeken hebben gevonden dat patiënten die getransplanteerd waren met een donorlever die genetisch onvoldoende MBL had meer bacteriële infecties na LTx doormaakten, maar een vierde onderzoek vond deze associatie niet. De drie onderzoeken die de associatie wel vonden, gebruikten echter alledrie een verschillende methode om donorlevers in te delen in *voldoende* of *onvoldoende* MBL. In ons onderzoek hebben we daarom donorlevers ingedeeld op basis van deze drie methoden, maar met geen enkele van deze drie methoden vonden we een associatie tussen genetische variatie in MBL van de donor en het optreden van infecties in LTx patiënten.

In **Hoofdstuk 4** hebben we onderzocht of er een associatie is tussen genetische variaties in diverse receptoren van het innate immuunsysteem van zowel donor als ontvanger en het optreden van infecties en afstoting in patiënten na LTx. Onze resultaten lieten zien dat geen enkele van de 50 geteste variaties was geassocieerd met het optreden van infecties of afstoting, terwijl een aantal van deze variaties in eerdere onderzoeken wel een verhoogd risico op infecties gaf in niet-transplantatiepatiënten met andere aandoeningen. Blijkbaar spelen na transplantatie andere dan genetische factoren een grotere rol in het risico op infecties en afstoting.

Discussie en handreikingen voor vervolgonderzoek

Aangezien ongeveer 80% van de LTx patiënten tenminste één episode van infectie doormaakt tijdens het eerste jaar na LTx en ongeveer 40% van de patiënten op de wachtlijst voor LTx één of meerdere infecties krijgt, is het belangrijk om risicofactoren voor infecties in deze patiënten te identificeren. In Deel I van dit proefschrift hebben we onderzocht of genetische variaties in het innate immuunsysteem risicofactoren kunnen zijn voor infecties in deze patiënten. Het innate immuunsysteem speelt namelijk een belangrijke rol bij de afweer tegen infecties en variaties in dit immuunsysteem zijn in andere patiëntengroepen geassocieerd met verschillen in risico's op infecties.

Zoals hierboven beschreven, vonden we in patiënten op de wachtlijst vóór LTx een sterke associatie tussen een genetische variatie in het TNF-α gen en infecties (**Hoofdstuk 2**), terwijl we in de **Hoofdstukken 3 en 4** geen associatie vonden tussen andere genetische variaties en infecties ná LTx. De vraag die dan rijst, is: waarom vonden we geen associatie

tussen diverse genetische variaties en infecties ná LTx terwijl andere onderzoeken die wel hebben gevonden en terwijl we ook een associatie vonden met infecties vóór LTx?

Ten eerste, in **Hoofdstuk 3** hebben we laten zien dat andere studies die wel een associatie vonden tussen genetische variaties in donor MBL en de kans op infecties na LTx verschillende methoden hebben gebruikt om donorlevers in te delen in *voldoende* of *onvoldoende* MBL op basis van de genetische variaties. Het is echter arbitrair welke methode gebruikt dient te worden en mogelijk waren deze assocaties niet gevonden als de onderzoekers andere methoden hadden gebruikt. Echter, met geen van de drie methoden hebben wij de associatie gevonden. Dit zou kunnen liggen aan de kortere follow-up tijd die wij hebben gebruikt om infecties in deze patiënten in kaart te brengen, terwijl in de andere studies een langere follow-up tijd werd genomen. Dit geldt ook voor de variaties die we in **Hoofdstuk 4** hebben onderzocht: in andere studies naar deze variaties werd vaak een follow-up van 1 jaar genomen, terwijl wij hebben gekeken in de eerste 3 maanden na LTx. Hiermee zouden we associaties hebben kunnen gemist.

Ten tweede, in **Hoofdstuk 4** hebben we laten zien dat genetische variaties in diverse receptoren van het innate immuunsysteem niet geassocieerd waren met infecties na LTx, terwijl in andere studies een aantal van deze variaties significant geassocieerd bleken te zijn met infecties in ernstig zieke niet-levertransplantatiepatiënten. Een reden voor dit verschil zou kunnen zijn dat de associaties die eerdere studies vonden vaak gevonden werden in receptoren die een *bepaalde groep* bacteriën herkent, de zogenaamde gram-positieve of juist gram-negatieve bacteriën. Hoewel wij in **Hoofdstuk 4** infecties ook hebben opgesplitst in infecties met gram-positieve versus gram-negatieve bacteriën, vonden wij niet dezelfde associaties als de andere onderzoeken. Dit zou temaken kunnen hebben met het gebruik van selectieve darmdecontaminatie (SDD), een methode die in LTx patiënten vóór het jaar 2000 gebruikt werd om de bacteriën in het maagdarmkanaal te verminderen, zodat deze patiënten mogelijk minder kans op infecties hadden. Er is echter later aangetoond dat SDD-gebruik bij LTx patiënten het risico op infecties in het algemeen niet beïnvloedt, maar dat er in verhouding wel meer infecties met gram-positieve bacteriën dan gram-negatieve bacteriën optreden. Doordat wij de groep patiënten met en zonder SDD-gebruik als één groep hebben geanalyseerd, hebben we mogelijk associaties tussen genetische variaties en het optreden van infecties gemist.

Een ander verschil tussen LTx patiënten en andere ernstig zieke patiëntencategorieën is dat LTx patiënten immunosuppressieve medicijnen gebruiken om afstoting van de donorlever te voorkomen. Het is echter onbekend of deze medicijnen ook invloed hebben op expressie van de receptoren van het innate immuunsysteem die we hebben onderzocht in **Hoofdstuk 4**. Wanneer dit zo is, zou dit kunnen verklaren waarom genetische variaties in deze receptoren wel geassocieerd zijn met infecties in niet-LTx patiënten, terwijl ze minder belangrijk zijn in LTx patiënten.

Verder, het is bekend dat infecties in verschillende tijdsperioden na LTx worden beïnvloed door verschillende klinische risicofactoren. Infecties in de eerste maand post-LTx worden sterk beïnvloed door chirurgische factoren en andere transplantatie-gerelateerde factoren, terwijl infecties tussen één en zes maanden na LTx meer gerelateerd zijn aan het gebruik van immunosuppressieve medicijnen en late infecties (meer dan zes maanden na LTx) kunnen worden beïnvloed door aanwezigheid van andere aandoeningen, zoals hepatitis C, diabetes en vermindere functie van de donorlever. Door de kortere follow-up tijd in onze studie (zie boven) zou het zo kunnen zijn dat de invloed van chirurgische en andere transplantatie-gerelateerde factoren op infecties groter is in onze studie dan in de andere studies, waardoor een significante invloed van genetische factoren gemist is. Hoewel we een aantal belangrijke transplantatie-gerelateerde factoren wel hebben meegenomen in de analyses, hebben we helaas een aantal andere factoren die van invloed kunnen zijn niet meegenomen, zoals lang verblijf op de Intensive Care, hoeveelheid bloedtransfusie tijdens LTx en scores die de mate van leverziekte voor LTx vastleggen.

Om in vervolgonderzoek verder te onderzoeken of genetische variaties in receptoren van het innate immuunsysteem het risico op infecties na LTx beïnvloeden, zouden we allereerst aanbevelen om een langere follow-up tijd te nemen en infecties in de eerste maand na LTx apart te analyseren van infecties die tussen de eerste en de zesde maand na LTx vóórkomen. Ten tweede zouden de hierboven genoemde klinische factoren meegenomen moeten worden in de analyses, aangezien deze het risico op infecties ook sterk kunnen beïnvloeden. Ten derde, grotere patiëntencohorten zijn nodig om het mogelijk te maken ook in subgroepen van LTx patiënten naar de invloed van genetische variaties op het risico op infecties te kijken, zoals de hierboven beschreven subgroepen van LTx patiënten die wel of geen SDD hebben ondergaan.

Een ander interessant onderwerp voor vervolgonderzoek is om de genetische variatie in het TNF-α gen die in **Hoofdstuk 2** in patiënten vóór LTx sterk geassocieerd was met infecties ook te analyseren in patiënten ná LTx. Hiervoor zouden dezelfde patiënten als in Hoofdstuk 2 gevolgd kunnen worden na hun LTx, waarbij in kaart gebracht wordt of de A-variatie in het TNF-α gen ook beschermend werkt tegen het optreden van infecties ná LTx. Omgekeerd zou het interessant zijn om te onderzoeken of de variaties die we in de **Hoofdstukken 3 en 4** hebben onderzocht ná LTx, in dezelfde patiënten voordat ze hun LTx ondergingen geassocieerd zijn met het optreden van infecties.

Ten slotte zou het meerwaarde kunnen hebben om de verschillende genetische variaties in het innate immuunsysteem te combineren met elkaar of met klinische factoren, om zo te onderzoeken of bepaalde combinaties een verhoogd of juist verlaagd risico geven op infecties. Dit zou voor de klinische praktijk een grotere voorspellende waarde voor het optreden van infecties kunnen hebben, waardoor patiënten kunnen worden

geïdentificeerd die meer of juist minder prophylactisch antibitiotica nodig hebben voor of na hun LTx.

II. Virale factoren die anti-donor T-cel reactiviteit beïnvloeden

Samenvatting

In Deel II (**Hoofdstukken 5-6**) hebben we onderzocht wat de invloed is van virale factoren, voornamelijk van cytomegalovirus (CMV) infectie, op de T-cel respons na LTx.

In **Hoofdstuk 5** hebben we gevonden dat $CD8^+$ T-cellen van patiënten met een primaire CMV infectie na LTx een verminderde respons hadden tegen donor materiaal (*donor-specifieke hyporesponsiviteit*). Verder vonden we dat in een groot cohort van LTx patiënten, getransplanteerd in ons centrum, patiënten met een primaire CMV infectie beschermd waren tegen het optreden van late acute afstoting. Ook hebben we in een deel van de LTx patiënten de aantallen Vδ1 en Vδ2 γδ T-cellen gemeten en de ratio tussen deze twee celtypen berekend. We vonden dat LTx patiënten met primaire CMV infectie hogere Vδ1/Vδ2 T-cel ratio's hadden dan LTx patiënten met een andere CMV serostatus. In eerdere studies waren hogere Vδ1/Vδ2 T-cel ratio's geassocieerd met operationele tolerantie. Wij concluderen daarom uit ons onderzoek dat primaire CMV infectie tolerantie voor de donorlever zou kunnen bevorderen.

In **Hoofdstuk 6** hebben we onderzocht of allogene T-cel responsen na LTx worden gereguleerd door aanwezigheid van co-inhibitoire receptoren. Het is namelijk bekend dat continue aanwezigheid van bijvoorbeeld virale infecties de expressie van co-inhibitoire receptoren op T-cellen kan verhogen en daardoor de T-cel respons tegen geïnfecteerd weefsel remt. Aangezien er na LTx ook continue aanwezigheid van lichaamsvreemd materiaal (*allo-antigenen*) is, was onze hypothese dat na LTx expressie van co-inhibitoire receptoren op T-cellen van LTx patiënten verhoogd is en dat daardoor T-cel respons tegen de donorlever geremd wordt. In dierexperimentele onderzoeken is aangetoond dat co-inhibitoire receptoren inderdaad tolerantie voor de donorlever kunnen bevorderen, maar of dit ook zo is in mensen is onbekend. In dit hoofdstuk hebben we laten zien dat, al zes maanden na LTx, de expressie van de co-inhibitoire receptoren CD160 en CD244 op circulerende $CD8^+$ T-cellen van LTx patiënten hoger was dan vóór LTx. Ook in patiënten lang na LTx was deze verhoging zichtbaar, met vooral een grote stijging van CD244. Interessant genoeg vonden we de sterke CD244-verhoging vooral in patiënten die een CMV infectie na LTx hadden doorgemaakt. Daarnaast was CMV infectie geassocieerd met verminderde deling en effector respons (cytotoxische degranulatie) van $CD8^+$ T-cellen in reactie op stimulatie met allo-antigenen laat na LTx. Daarnaast deelden opgezuiverde $CD244^+CD8^+$ T-cellen van LTx patiënten minder goed dan CD244-$CD8^+$ T-cellen

in reactie op stimulatie met allo-antigenen of een polyclonale stimulus. We zagen ook dat de $CD244^+CD8^+$ T-cel populatie de meerderheid van CMV-tetrameer positieve cellen bevatte. Concluderend hebben we gevonden dat CMV infectie na LTx is geassocieerd met toename van een dysfunctionerende T-cel subset, namelijk $CD244^+CD8^+$ T-cellen, en met allogene hyporesponsiviteit van $CD8^+$ T-cellen. Deze resultaten suggereren dat CMV infectie $CD8^+$ T-cel responsen na LTx inperken.

Discussie en handreikingen voor vervolgonderzoek

T-cel responsen tegen virussen, onder anderen tegen CMV, zijn in eerdere onderzoeken beschreven als één van de belangrijkste barrières voor tolerantie voor een donororgaan. Dit komt waarschijnlijk doordat T-cellen die tegen virussen zijn gericht kruisreageren tegen allogeen HLA van het donororgaan, leidend tot verhoogde T-cel reacties tegen het donororgaan en dus meer kans op afstoting. In tegenstelling tot deze bevindingen, hebben wij in de **Hoofdstukken 5 en 6** laten zien dat CMV infectie na LTx juist geassocieerd was met lágere T-cel responsen, hetgeen eerder tot tolerantie dan tot afstoting zou leiden. In **Hoofdstuk 5** toonden we dit aan doordat we in patiënten met primaire CMV infectie na LTx lagere T-cel responsen waarnamen, tezamen met de klinische bevestiging in een groot patiëntencohort dat LTx patiënten met een primaire CMV infectie minder acute afstoting hadden. Daarnaast vonden we in deze patiëntencategorie hogere ratio's Vδ1/Vδ2 T-cellen, iets wat in eerdere studies geassocieerd was met tolerantie voor donorlevers. **Hoofdstuk 6** voegde hieraan een meer mechanistisch aspect toe, namelijk de bevinding dat CMV infectie een verhoogde expressie van de co-inhibitoire receptor CD244 induceert op $CD8^+$ T-cellen van LTx patiënten. De cellen die CD244 tot expressie brengen bleken vervolgens minder sterk te reageren op stimulatie met zowel allo-antigenen als een polyclonale stimulus en daarnaast bleek deze celpopulatie de meerderheid van de CMV-specifieke T-cellen te bevatten in patiënten met CMV infectie na LTx. Tezamen laten deze data zien dat CD244-inductie één van de mechanismen kan zijn waardoor CMV infectie na LTx leidt tot lagere T-cel responsen tegen allo-antigen. Echter, wanneer we CD244 blokkeerden met een antistof waardoor het effect van CD244 wordt tegengegaan, gingen de T-cellen niet sterker reageren. We concluderen daarom dat CD244 expressie op T cellen van CMV-geïnfecteerde LTx patiënten eerder een surrogaatmarker voor expansie van gedifferentieerde cellen met verminderde functie is, dan het mechanisme zelf dat leidt tot tolerantie.

Hoewel de bevindingen van de **Hoofdstukken 5 en 6** met elkaar in lijn zijn, is er ook een aantal verschillen tussen deze hoofdstukken. Ten eerste, in **Hoofdstuk 5** hebben we T-cel responsen in patiënten vroeg na LTx bepaald, terwijl we in **Hoofdstuk 6** patiënten laat na LTx hebben bestudeerd. Daarom kunnen de twee hoofdstukken niet één op één vergeleken worden.

Een tweede verschil tussen de hoofdstukken is dat we twee verschillende assays hebben gebruikt om responsen van alloreactieve $CD8^+$ T-cellen te meten: in **Hoofdstuk 5** keken we naar de expressie van een vroege activatiemarker (na 24 uur stimulatie), terwijl we in **Hoofdstuk 6** deling en effector functie van T-cellen maten (na 5 dagen stimulatie). Interessant genoeg vonden we hiermee dat de vroege activatie van alloreactieve $CD8^+$ T-cellen donor-specifiek verlaagd was in CMV-geïnfecteerde patiënten (**Hoofdstuk 5**) en dat ook de deling van alloreactieve $CD8^+$ T-cellen donor-specifiek verlaagd was (**Hoofdstuk 6**). Echter, de effector functie van alloreactieve $CD8^+$ T-cellen was in CMV patiënten verlaagd tegen niet alleen donor allo-antigenen maar ook allo-antigenen van andere origine. Dit zou erop kunnen wijzen dat $CD8^+$ T-cellen in CMV patiënten na LTx *in het algemeen* uitgeput raken en minder goed functioneren en niet alleen in reactie op allo-antigenen van de donor. Het is onbekend waardoor dit komt. Om dit te onderzoeken zou een microarray experiment gedaan kunnen worden op $CD8^+$ T-cellen met en zonder CD244, om te onderzoeken of er verschillen in genexpressie zijn tussen deze twee cel-populaties die zouden kunnen verklaren waarom de ene populatie sterker reageert dan de andere.

Een derde verschil tussen de twee hoofdstukken is dat in **Hoofdstuk 5** de associatie tussen CMV infectie en lagere T-cel responsen alleen werd gevonden in LTx patiënten met een primaire CMV infectie, terwijl in **Hoofdstuk 6** deze associatie werd gevonden in zowel patiënten met een primaire CMV infectie als een reactivatie van het virus na LTx. In **Hoofdstuk 5** werden patiënten met en zonder reactivatie van het virus juist als één groep beschouwd. Dit heeft temaken met het feit dat in veel patiënten na LTx niet wordt gediagnosticeerd of er reactivatie is of niet, omdat dit vaak klinisch niet relevant lijkt te zijn. Om beter te onderzoeken of de beschreven associatie ook geldt voor de groep met CMV-reactivatie, zou in de toekomst een studie moeten worden gedaan waarbij in alle patiënten na LTx diagnostiek wordt verricht om te onderzoeken of er reactivatie van het virus is of niet. Alleen dan kan een volledig correct onderscheid gemaakt worden tussen patiënten met en zonder reactivatie van CMV.

Een ander interessant onderwerp voor vervolgonderzoek is de link tussen CMV infectie en operationele tolerantie na LTx. De hierboven beschreven bevindingen suggereren wel dat CMV infectie leidt tot tolerantie, maar om dit echt aan te tonen is een prospectieve trial met LTx patiënten nodig bij wie immunosuppressieve medicijnen worden afgebouwd en T-cel responsen worden gemeten op verschillende tijdstippen, tezamen met het bepalen van de CMV status. Wanneer inderdaad blijkt dat CMV infectie leidt tot meer tolerantie na LTx (geen afstoting van de donorlever terwijl de patiënt geen immunosup-pressieve medicijnen gebruikt), dan zou CMV status door artsen als factor meegenomen kunnen worden om te bepalen of de immunosuppressieve medicijnen kunnen worden

afgebouwd of niet. Daarnaast is vervolgonderzoek in het laboratorium nodig om te onderzoeken wat de oorzaak is dat CMV infectie leidt tot lagere T-cel responsen.

III. Optimalisatie van immuunsuppressie na levertransplantatie

Samenvatting

Na LTx worden patiënten behandeld met immunosuppressiva om afstoting van het levertransplantaat te voorkomen. In **Hoofdstuk 7** van dit proefschrift hebben we beschreven dat sinds de jaren '80 calcineurine remmers zoals cyclosporine A (CsA) en tacrolimus (TAC) de meest gebruikte immunosuppressiva zijn na LTx. Deze medicijnen remmen calcineurine, wat via een signaal in de cel leidt tot remming van allerlei transcriptiefactoren, resulterend in verminderde activatie van genen betrokken bij interleukine (IL)-2, TNF-α, IL-3, IL-4, *granulocyte-macrophage colony-stimulating factor* en IFN-γ. Uiteindelijk leidt dit tot verminderde deling van T-cellen en verminderde productie van pro-inflammatoire cytokinen. Door remming van de T-cel reactiviteit wordt afstoting van het transplantaat voorkomen. Echter, doordat de T-cel reactiviteit wordt geremd, die ook nodig is om ziekteverwekkers aan te vallen, leidt het gebruik van calcineurine remmers tot een verhoogd risico op ernstige infecties. Andere bijwerkingen van calcineurine remmers zijn nierschade, zenuwschade, hoge bloeddruk, kanker en diabetes mellitus. Toen in de jaren '90 aangetoond werd dat TAC geassocieerd was met minder nierschade en zenuwschade dan CsA werd TAC het eerste keus immunosuppressieve middel na LTx, vaak gecombineerd met prednisolon. Echter, nog steeds kunnen er allerlei bijwerkingen optreden van het gebruik van immunosuppressiva en daarom zijn er strategieën bedacht om de dosering van TAC te minimaliseren. Vier van deze strategieën zijn beschreven in Hoofdstuk 7:

1. *Switch naar een immunosuppressief schema zonder calcineurine remmers als er schade door calcineurine remmers is opgetreden*
2. *Start direct na LTx al met een mTOR remmer in combinatie met andere immunosuppressiva (schema zonder calcineurine remmer)*
3. *Vertraagde introductie van tacrolimus in lagere dosering, gecombineerd met mycofenolaat mofetil en dacluzimab*
4. *Switch naar een immunosuppressief schema zonder calcineurine remmers vroeg na LTx*

In **Hoofdstuk 8** was onze hypothese dat de immunosuppressieve eigenschappen van mesenchymale stamcellen (MSCs) uit donorlevers (L-MSCs) sterker zijn dan die van mesenchymale stamcellen uit beenmerg (BM-MSCs). Van beide typen MSCs hebben we daarom onderzocht hoe sterk ze allogene T-cel responsen remmen om deze vervolgens

te vergelijken. Hiervoor isoleerden we allereerst MSCs uit perfusaten (spoelvloeistoffen) van donorlevers en kweekten deze op om ze in aantal te laten toenemen. Toen we beide typen MSCs aan reacties van allogene T-cellen toevoegden, zagen we dat L-MSCs de deling en effector functie (cytotoxische degranulatie en IFN-γ productie) van allogene T-cellen veel sterker remden dan BM-MSCs. Deze remming kwam tot stand door zowel cel-cel contact als door MSCs uitgescheiden factoren. Dit laatste concludeerden we op basis van de bevinding dat het kweekmedium waarin de L-MSCs gekweekt waren ook in staat was om T-cel repsonsen te remmen, terwijl kweekmedium van BM-MSCs geen remming veroorzaakte. Verder zagen we dat L-MSCs al na isolatie uit de donorlevers een hogere expressie van PD-L1 vertoonden dan BM-MSCs, wat geassocieerd was met sterkere remming van T-cel responsen. Wanneer we PD-L1 vervolgens blokkeerden, werd de remming van cytotoxische degranulatie die L-MSCs bewerkstelligden deels opgeheven. Verder zagen we dat blokkeren van de factor indolamine-2,3-dioxygenase (IDO) de remmende effecten van L-MSCs op T-cel deling ook deels kon opheffen, terwijl dit geen effect had op de condities met BM-MSCs. Concluderend, L-MSCs onderdrukken allogene T-cel responsen sterker dan BM-MSCs, wat gerelateerd kan zijn aan de condities in de donorlever tijdens de donatie. Deze L-MSCs zouden relevant kunnen zijn voor het onderdrukken van ontvanger T-cel responsen tegen het levertransplantaat.

Discussie en handreikingen voor vervolgonderzoek

In **Hoofdstuk 7** hebben we beschreven dat calcineurine remmers nog steeds de hoeksteen zijn van immunossuppressieve therapie na LTx, ondanks hun bijwerkingen. Om de bijwerkingen van deze groep medicijnen te minimaliseren, hebben we een aantal strategieën voorgesteld om het gebruik te reduceren of te vervangen door andere type medicijnen. Een mogelijke kandidaat is het gebruik van mTOR remmers, waarvan gedacht wordt dat bijwerkingen als nierschade en kanker minder zijn dan met het gebruik van calcineurine remmers. In een groot onderzoek, waaraan ook ons centrum deelnam, is aangetoond dat in patiënten die een combinatie van een calcineurine remmer en een mTOR remmer (Everolimus genaamd) gebruikten, de nierfunctie beter was dan in patiënten die alleen een calcineurine remmer gebruikten. Een andere studie waarbij een vergelijkbaar middel wordt onderzocht is op dit moment nog gaande en de resultaten hiervan kunnen over een aantal jaar worden verwacht. Verder onderzoek is nog steeds nodig om het gebruik van calcineurine remmers en daarmee hun bijwerkingen zoveel mogelijk te beperken.

Naast klinische studies die erop gericht zijn om bijwerkingen van immunosuppressieve medicijnen te beperken, is er basaal onderzoek nodig om alternatieve behandelingsstrategieën te ontwikkelen die in de toekomst mogelijk immunosuppressieve medicijnen compleet kunnen vervangen. Voor dat doel wordt veel onderzoek gedaan naar behan-

deling met cellen in plaats van medicijnen. Eén kandidaat hiervoor is behandeling met mesenchymale stamcellen (MSCs), een celtype dat een goede kandidaat zou zijn vanwege zijn afweeronderdrukkende capaciteiten. MSCs komen voor in allerlei weefsels, waaronder beenmerg en lever. Ook uit donorlevers kunnen deze cellen worden geïsoleerd. In **Hoofdstuk 8** hebben we voor het eerst de afweeronderdrukkende capaciteiten vergeleken van MSCs die uit beenmerg komen versus die uit donorlevers komen. Hierbij toonden we aan dat MSCs uit donorlevers T-cel responsen veel sterker onderdrukken dan MSCs uit beenmerg. Aangezien T-cel responsen afstoting kunnen veroorzaken, zou deze bevinding kunnen betekenen dat MSCs uit levers afstoting tegen kunnen gaan en dat ze dit nog sterker kunnen dan MSCs uit beenmerg.

Om therapie met MSCs na transplantatie praktisch mogelijk te maken, moet er nog wel een aantal vervolgstappen gedaan worden. Allereerst moeten MSCs worden geïsoleerd uit weefsel, bijvoorbeeld beenmerg of lever. De cellen moeten daarna worden vermenigvuldigd in het laboratorium, waarna ze intraveneus kunnen worden toegediend aan de patiënt. Hoewel eerdere studies hebben laten zien dat na toediening de cellen maar kort leven en in de longen van de patiënt blijven hangen, hebben studies bij niertransplantatiepatiënten laten zien dat deze methode veilig is en dat de cellen hun afweeronderdrukkende effect in korte tijd al hebben uitgevoerd. Waarschijnlijk doen ze dit door middel van een afweeronderdrukkend effect op andere celtypen die wel langer in leven blijven.

Een aantal zaken betreffende MSC-therapie na transplantatie behoeven vervolgonderzoek:
- Optimale timing van MSC-infusie in de patiënt, bijvoorbeeld tijdens de transplantatie of enige weken erna
- Wel of geen gebruik van andere immunosuppressieve medicijnen naast de MSCs
- Oncogeniciteit van MSCs en het eventueel optreden van opportunistische infecties
- Uit welk weefsel MSCs geïsoleerd dienen te worden

Dit laatste onderwerp is sterk gerelateerd is aan het onderzoek dat we in **Hoofdstuk 8** hebben verricht, aangezien we in dit hoofdstuk de immunosuppressieve capaciteit van lever-MSCs versus beenmerg-MSCs hebben vergeleken. Enerzijds zou het gebruik van beenmerg-MSCs voordeel kunnen hebben ten opzichte van MSCs uit donorlevers, omdat beenmerg-MSCs geïsoleerd kunnen worden uit gezonde individuen (beenmerg donoren), waardoor een voorraad MSCs voor therapie opgeslagen kan worden die direct beschikbaar is op het benodigde moment (tijdens transplantatie). Gebruik van MSCs uit donorlevers brengt met zich mee dat deze eerst moeten worden vermenigvuldigd, waardoor ze pas enige tijd na LTx beschikbaar zijn voor toediening aan de patiënt. Een voordeel zou echter kunnen zijn dat kleinere aantallen cellen mogelijk al tot het gewenste

immunosuppressieve effect leiden, aangezien we in **Hoofdstuk 8** hebben aangetoond dat lever-MSCs T-cel responsen veel sterker onderdrukten dan beenmerg-MSCs. Dit laatste zou echter nog verder onderzocht moeten worden.

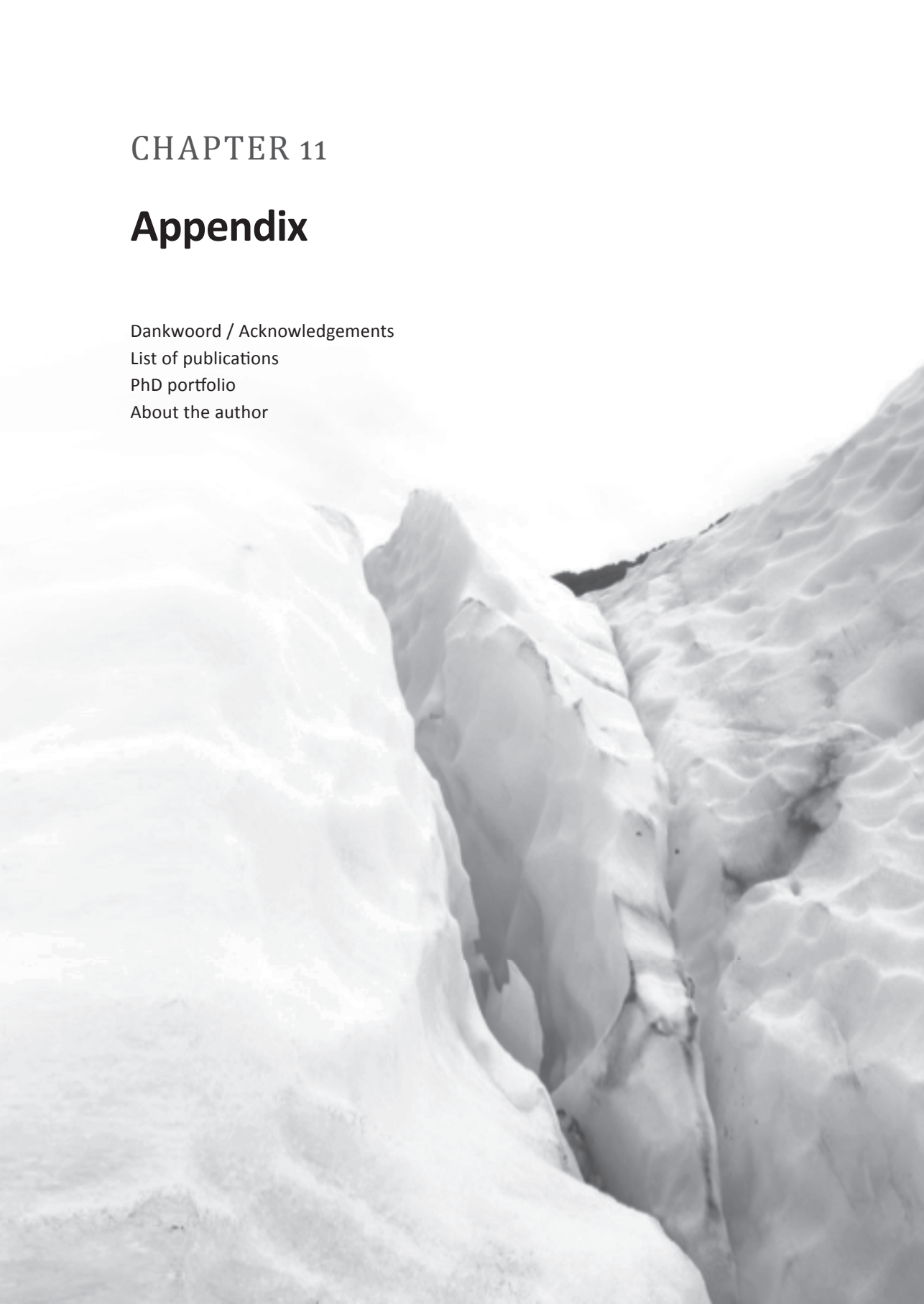
Conclusie

In dit proefschrift hebben we beschreven dat genetische en virale factoren kunnen bijdragen aan het bepalen van het risico op complicaties zoals infecties en afstoting in LTx patiënten. De rol van genetische factoren in het ontstaan van infecties lijkt echter vóór LTx groter te zijn dan ná LTx. Daarnaast hebben we klinische strategieën beschreven die het gebruik van immunosuppressieve medicijnen na LTx kunnen optimaliseren, waardoor bijwerkingen van deze medicijnen beperkt kunnen worden. Ook hebben we basale bevindingen gedaan die ertoe kunnen leiden dat in de toekomst het gebruik van de huidige immunosuppressieve medicijnen na LTx kunnen worden vervangen door therapie met mesenchymale stamcellen, die afstotingsreacties sterk kunnen onderdrukken.

CHAPTER 11

Appendix

Dankwoord / Acknowledgements
List of publications
PhD portfolio
About the author



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dat dit minder liep dan gehoopt, ik hoop dat dit in de toekomst een mooier vervolg zal krijgen.

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Emmeloes

LIST OF PUBLICATIONS

1. Curvelo LA, de Mare-Bredemeijer E, de Canck I, van Thielen M, Kazemier G, Metselaar H, Kwekkeboom J. Does the donor mannose-binding lectin genotype really predict the risk of bacterial infections after liver transplantation? Hepatology. 2011 May;53(5):1786-7.

2. de Mare-Bredemeijer EL, Metselaar HJ. Optimization of the use of Calcineurin inhibitors in liver transplantation. Best Pract Res Clin Gastroenterol. 2012 Feb;26(1):85-95.

3. de Mare-Bredemeijer EL, Mancham S, Utomo WK, de Canck I, van Thielen M, de Meester E, Rossau R, van der Laan LJ, Hansen BE, Tilanus HW, Kazemier G, Janssen HL, Metselaar HJ, Kwekkeboom J. Genetic polymorphisms in innate immunity receptors do not predict the risk of bacterial and fungal infections and acute rejection after liver transplantation. Transpl Infect Dis. 2013 Apr;15(2):120-33.

4. Renáta Senkerikova*, Emmeloes de Mare-Bredemeijer*, Soňa Fraňková, Dave Roelen, Thijmen Visseren, Pavel Trunečka, Julius Špičák, Herold Metselaar, Milan Jirsa, Jaap Kwekkeboom, Jan Šperl. Genetic variation in TNFA predicts protection from severe bacterial infections in patients with end-stage liver disease awaiting liver transplantation. J Hepatol 2013 Dec 18. pii S0168-8278(13)00879-9. **shared first authorship*

5. X-L. Shi, E.L.D. de Mare-Bredemeijer, Ö. Tapirdamaz, B.E. Hansen, R. van Gent, M.J.H. van Campenhout, S. Mancham, N.H.R. Litjens, M.G.H. Betjes, A.A. van der Eijk, Q. Xia, L.J.W. van der Laan, J. de Jonge, H.J. Metselaar, J. Kwekkeboom CMV primary infection is associated with donor-specific T-cell hyporesponsiveness and fewer late acute rejections after liver transplantation. *Am J Transplant 2015; doi: 10.1111/ajt. 13288*

6. Emmy L.D. de Mare-Bredemeijer, Shanta Mancham, Monique M.A. Verstegen, Petra E. de Ruiter , Rogier van Gent, David O'Neill, Hugo W. Tilanus, Herold J. Metselaar, Jeroen de Jonge, Jaap Kwekkeboom, Sean R.R. Hall[2]* and Luc J.W. van der Laan* Human liver graft-derived mesenchymal stromal cells potently suppress allo-reactive T-cell responses. Stem Cells and Development 2015 *Stem Cells Dev. 2015; 24(12): 1436-47 *Shared last authorship*

7. E.L.D. de Mare-Bredemeijer, X-L. Shi, S. Mancham, R. van Gent, S.A. van der Heide-Mulder, R. de Boer, M.H.M. Heemskerk, J. de Jonge, L.J.W. van der Laan, H.J. Metselaar, J. Kwekkeboom CMV-induced expression of CD244 after liver transplantation

is associated with CD8+ T-cell hyporesponsiveness to allo-antigen. *Accepted for publication in The Journal of Immunology*

PHD PORTFOLIO

Name PhD student	Emmy Louise Dorothea (Emmeloes) de Mare-Bredemeijer
Erasmus MC Department	Gastroenterology and Hepatology
PhD period	January 2011 – December 2014
Promotor	Prof. dr. H.J. Metselaar
Co-promotor	Dr. J. Kwekkeboom

General courses

Februari 2014	Biomedical English writing and Communication
Maart 2013	Biostatistical Methods I: Basic Principles (CC02a), NIHES, Rotterdam
Maart 2012	The advanced Course "Molecular Immunology", Rotterdam
Oktober 2010	Good Clinical Practice / Basiscursus regelgeving en organisatie voor klinisch onderzoekers, Rotterdam
September 2010	Short Introductory Course on Statistics & Survival Analysis for MD's, Rotterdam

National conferences – Presentations

2014	NVVI 50th Anniversary Congress 2014, Efteling, Kaatsheuvel, The Netherlands (poster presentation)
2013	Bootcongres, NTV, Duiven, The Netherlands (oral presentation)
2013	Voorjaarscongres 2013 NVGE, Veldhoven, The Netherlands (poster presentation)

International conferences – Presentations

2014	World Transplant Congress 2014, San Francisco, USA (poster presentation)
2013	American Transplantation Congress, Seattle, USA (oral presentation)
2013	International Liver Congress 2013, EASL, Amsterdam, The Netherlands (oral presentation)
2013	ILTS 2013, 19th Annual International Congress, Sydney, Australia (poster presentation)
2012	ILTS 2012, 18th Annual International Congress, San Francisco, USA (poster presentation)

National conferences – Participation

2012	APC's revisited, the function of antigen presenting cells in health and disease, NVVI, Lunteren, The Netherlands
2012	Bootcongres, NTV, Maastricht, The Netherlands
2011	Bootcongres, NTV, Amsterdam, The Netherlands
2011	Time for high T: Features and functions of T-cells in health and disease, NVVI, Lunteren, The Netherlands

International conferences – Participation

2011	The 2nd International Conference on Transplantomics and Biomakers in Organ Transplantation, Barcelona, Spain

Scientific Awards and Grants

2013	Registration Bursary EASL International Liver Congress 2013 and free membership EASL
2013	Travel grant Astellas Pharma B.V.
2013	Travel grant Dutch Society of Hepatology (NVH)
2013	Travel Grant Trustfonds Erasmus MC (for ATC 2013, Seattle)

Teaching activities

2012	Lecture Minor Transplantatiegeneeskunde 2012
2011-2012	Supervising students Master of Science Infection and Immunity

Other activities

2011-2014	Local study coordinator and co-investigator of "A multi-center randomized, open label, controlled study in primary liver transplantation comparing long term renal function in recipients treated with standard dose extended-release tacrolimus alone and recipients treated with a combination of low dose extended-release tacrolimus and low dose sirolimus"
2011-2013	Co-investigator of "Extension study to the multicenter, open-label, randomized, controlled study CRAD001H2304 to evaluate the long-term efficacy and safety of concentration controlled everolimus in liver transplant recipients"

ABOUT THE AUTHOR

Emmeloes de Mare-Bredemeijer was born as Emmy Louise Dorothea Bredemeijer on April 7th 1986 in Harderwijk, The Netherlands. She was raised by her beloved parents Ferdi Bredemeijer and Emmy Cnossen, and grew up together with her 4 sisters and 3 brothers. In 2004 she completed secondary school and started her medical school at the Erasmus University Rotterdam, The Netherlands. In 2008, she performed her graduation research in the Laboratory of Gastroenterology and Hepatology, under supervision of Dr. Jaap Kwekkeboom and Prof. Dr. Herold Metselaar. Her research was entitled "Genetic polymorphisms in innate immune receptors as a risk factor for rejection and infection after liver transplantation". After two years of clinical internships, she obtained her medical degree in 2010, and started her PhD project. During this project, she combined clinical research on immunosuppression in liver transplantation patients with basic research in the laboratory at the Department of Gastroenterology and Hepatology of the Erasmus University Medical Center Rotterdam, again under supervision of Dr. Jaap Kwekkeboom and Prof. Dr. Herold Metselaar. In 2013, Emmeloes and her husband Rutger de Mare received a son: Felix. In May 2015 their second son was born: Victor. After finishing her PhD, Emmeloes is going to work as fertility doctor at Sint Franciscus Gasthuis Rotterdam.